University of Alberta

Studies on the Structure and Function of the *Saccharomyces cerevisiae* Succinate Dehydrogenase and its Chaperone Tcm62p

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Spring, 2008

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ISBN: 978-0-494-45614-9
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ISBN: 978-0-494-45614-9

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ABSTRACT

The mitochondrial succinate dehydrogenase (SDH) is an essential component of the electron transport chain and of the tricarboxylic acid cycle. Also known as complex II, this tetrameric enzyme catalyzes the oxidation of succinate to fumarate and reduces ubiquinone to ubiquinol. Because of its central role in cellular energy generation, SDH defects can manifest themselves as diseases with a diverse array of clinical presentations. SDH dysfunction can arise from mutations that disrupt function or defects in the biogenesis of the enzyme. The proper folding and assembly of complex cofactor-containing holoenzymes such as SDH is a significant and intricate process that requires the presence of assembly factors. Using the *Saccharomyces cerevisiae* SDH as a model, our aim is to understand its biogenesis and the molecular mechanisms of pathogenesis associated with SDH dysfunction.

We examined the structure and function of Tcm62p, an assembly factor for the yeast SDH. As a distant member of the Hsp60 family of molecular chaperones, it was proposed that Tcm62p might function as an ATPase. However, our evidence obtained through site-directed mutagenesis suggests that it is not. Tcm62p is an integral membrane protein as judged by the requirement for detergent to solubilize it from the membrane. We examined residues Glu-486 and Lys-490, thought to be present in the single transmembrane segment and show that they contribute partially to the carbonate extractability of Tcm62p. Our studies reveal that Tcm62p associates with other proteins to form two high molecular weight complexes. The presence of other proteins in these complexes suggests that there are additional assembly factors involved in SDH biogenesis but their identities remain unknown. We also investigated the relationship between Tcm62p and another mitochondrial chaperone, the prohibitin complex and showed that they function independently of each other.

Mutations in the human SDH genes are associated with the development of several types of tumors. However, the mechanisms of pathogenesis are still unclear. We investigated the molecular and catalytic effects of tumorigenic or tumor-related SDH mutations. We mutated Arg-47 of the Sdh3p subunit and Asp-88 of the Sdh4p subunit. Both residues are evolutionarily conserved and Arg-47 is a known site of tumorigenic mutations in humans. All mutants examined have reduced ubiquinone reductase activities. Several mutants are hypersensitive to oxidative stress and generate elevated levels of superoxide *in vitro* and *in vivo*. SDH mutations also cause the accumulation and secretion of succinate. Succinate can inhibit prolyl hydroxylase enzymes, resulting in a proliferative response mediated by the activation of hypoxia-inducible factor-1α. We suggest that certain SDH mutations can promote oncogenesis by contributing both to the production of reactive oxygen species and to a proliferative response normally induced by hypoxia but amplified by the accumulation of succinate.

For my mom, 姨媽 *and* 二舅母

Acknowledgements

This thesis is the culmination of a significant amount of hard work on my part but I could not have achieved this without the help and contributions of many people.

First and foremost, I have to thank my supervisor, Dr. Bernard Lemire for allowing me to pursue my doctoral studies in his laboratory. I am grateful for his support, patience, guidance and mentorship. I believe that under his tutelage, I learned what it takes to become a good scientist. I am also very appreciative of the fact he gave me the freedom to focus on areas of research that I found interesting.

Very special thanks to two of my former colleagues, Leanne Sayles and Dr. Les Grad. These two individuals made it fun to come to the lab to work every day and with them, the daily grind was a little more palatable. Although they were not directly involved in my research projects, these two individuals helped me a great deal in other capacities. I also want to thank Dr. William Tsang who was the graduate student that I followed during my summer studentships here in the Lemire lab; he was instrumental in my decision to enter into graduate school. I would like to thank Dr. Stephen Oyedotun for his for technical advice, Yuri Silkin for help in construction of the *SDH4* mutants, Dr. Brian Sykes for use of the NMR spectrometer, Drs. Joel Weiner and Dr. Richard Rothery for use of their lab equipment and Jeff DeVries and Angela Thiessen for technical support as well. A special thanks to Stacey Reinke who made significant contributions to my research by doing the metabolomic analysis on the SDH mutants. I would also like to thank other past and present members of the Lemire lab, especially my two summer students, Albert Woo and Wilson Chan. In addition, I would like to thank Barb Thom, our administrative assistant, for her tireless work during the preparation of my scholarship applications and for her help in preparing all the other paperwork that was required as a part of my graduate studies.

I am very grateful for the efforts of Drs. Howard Young and Joel Weiner who are the current members of my supervisory committee. I would also like to thank the former members of supervisory committee, Drs. Carlos Fernandez-Patron and Moira Glerum for their guidance, suggestions and advice throughout my Ph.D. studies. I would also like to thank Dr. Frank Nargang who acted as my internal external examiner. I am also very grateful for the efforts of Dr. Peter Thorsness who traveled here from Wyoming to be my external examiner. I also want to thank my fellow graduate students in the department who made this long journey a very memorable one. I have the utmost respect for them because I now know how much effort is required to achieve this goal. During my time here I have had the opportunity to meet some wonderful and interesting people. I believe that these interactions and experiences have enriched my life and made me a better person for it.

I am indebted to all my family and friends for their unending support in my pursuit of higher education. Everyone helped in their own way and this thesis is a testament to their belief in my abilities.

I am also extremely grateful for the financial support provided by Alberta Heritage Foundation for Medical Research, National Sciences and Engineering Research Council, University of Alberta and the Department of Biochemistry. The research described in this thesis was funded by a grant from the Canadian Institutes for Health Research.

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List of Abbreviations

ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
ANT	adenine nucleotide translocase
Apaf-1	apoptotic peptidase activating factor 1
ATP	adenosine triphosphate
BN-PAGE	blue native gel electrophoresis
COX	cytochrome *c* oxidase
CybL	cytochrome *b* large subunit of succinate dehydrogenase
CybS	cytochrome *b* small subunit of succinate dehydrogenase
DB	decylubiquinone
DCPIP	dichlorophenol indophenol
DDM	dodecyl-β-D-maltoside
DHE	dihydroethidium
DNA	deoxyribonucleic acid
EPR	electron paramagnetic spectroscopy
F_1	factor 1 of ATP synthase
FAD	flavin adenine dinucleotide
Fe-S	iron-sulfur cluster
FMN	flavin mononucleotide
F_O	factor oligomycin of ATP synthase
Fp	flavoprotein subunit of succinate dehydrogenase
FRD	fumarate reductase
HIF	hypoxia-inducible factor
IF_1	inhibitor factor 1
Ip	iron-sulfur protein subunit of succinate dehydrogenase
ISP	Rieske iron-sulfur protein
kDA	kilodaltons
mdm	mitochondrial distribution and morphology
mg	milligram
min	minutes
$mitoK_{ATP}$	mitochondrial ATP-sensitive potassium channel
mL	milliliter
mM	millimolar
MPP	mitochondrial processing peptidase
MPT	mitochondrial permeability transition pore
MRC	mitochondrial respiratory chain
mtDNA	mitochondrial DNA
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
OD_{600}	optical density at 600 nm
OSCP	oligomycin sensitivity-conferring protein
OXPHOS	oxidative phosphorylation
PAM	presequence translocase-associated motor

PBS	phosphate buffered saline
PCP	pentachlorophenol
PCR	polymerase chain reaction
PH	prolyl hydroxylase
PLD	phospholipase D
P_i	inorganic phosphate
PMS	phenazine methosulfate
Q	quinone
Q_D	distal quinone binding site
Q_P	proximal quinone binding site
RNA	ribonucleic acid
ROS	reactive oxygen species
SAM	sorting and assembly machinery
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
SQO	succinate-ubiquinone oxidoreductase
TCA	trichloroacetic acid
TIM	translocase of the mitochondrial inner membrane
TOM	translocase of the mitochondrial outer membrane
TTFA	thenoyltrifluoroacetone
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
VDAC	voltage-dependent anion channel, porin
$\Delta\psi_m$	mitochondrial inner membrane potential
Δp	proton motive force
μg	microgram
μL	microliter

CHAPTER 1

General Introduction

1.1 Introduction

The mitochondrial respiratory chain (MRC) plays a pivotal role in the energy metabolism of eukaryotic cells. It is comprised of a series of membrane bound cofactor-containing, multisubunit protein complexes. A wealth of information about the structure and function of these enzymes and their roles in cellular energy metabolism is available. However, the molecular mechanisms that govern the biogenesis of these intricate enzymes remain poorly understood. Elucidating the details of MRC complex assembly is important because assembly defects are associated with a large array of mitochondrial disorders. In addition, we also lack a detailed understanding of the molecular mechanisms of pathogenesis resulting from mitochondrial respiratory chain dysfunction. Using *Saccharomyces cerevisiae* as a model system, the work presented in this thesis examines the biogenesis of the succinate dehydrogenase (SDH) complex. We have examined the structure and function of a chaperone protein and investigated the molecular mechanisms of pathogenesis associated with SDH dysfunction. The aim of this chapter is to provide a brief overview of the general characteristics of mitochondria and the biological processes they are associated with. It will include a summary of the structure and function of the MRC, with a particular emphasis on SDH or complex II. This chapter will also discuss the association of mitochondrial respiratory chain dysfunction with human disease.

1.2 Mitochondria

1.2.1 *General Characteristics*

The ability to utilize nutrients to derive energy is a process fundamental to life. Cells have evolved structures and mechanisms to harness the chemical energy found in organic molecules and to utilize it for growth and reproduction. For eukaryotic cells, the structure that is responsible for the majority of cellular energy production is the mitochondrion. Mitochondria are essential organelles found in almost all eukaryotes. Eukaryotes that do not contain mitochondria possess alternative organelles such as mitosomes or hydrogenosomes, which are responsible for energy generation (Hackstein *et al.*, 2006).

The primary function of mitochondria is to provide energy for the cell through the production of adenosine triphosphate (ATP), the energy currency of the cell (Scheffler, 1999). Mitochondria are also involved in many other crucial metabolic pathways, including the Krebs cycle, β-oxidation of fatty acids, the urea cycle, iron-sulfur cluster assembly and the biosynthesis of heme, coenzyme Q (also known as ubiquinone) and lipids (Scheffler, 2000). The existence of mitochondria in their current form is the result of an endosymbiotic event that occurred approximately two billion years ago and resulted in a bacterium becoming a resident inside a primitive eukaryote (Gray *et al.*, 1999). This relationship evolved with the loss of redundant genes from the bacterium, while others were transferred to the genome of the host eukaryote; today, only a very small number of bacterial genes remain in the mitochondrial DNA (mtDNA). These events created the dual genome state observed in most eukaryotic cells today (Gray *et al.*, 2001).

Mitochondria resemble bacteria in their size and shape. They are visualized as sausage-like organelles in certain cell types, such as human hepatocytes and fibroblasts (Scheffler, 1999). Typically in those cell types, they have the dimensions of 3-4 μm in length and approximately 1 μm in diameter. The number of mitochondria per cell is highly variable with hepatocytes containing approximately 800 per cell, whereas a lower eukaryote such as the amoeba can have as many as 500,000 (Scheffler, 1999). In the higher eukaryotes, which contain more developed and specialized cells, the number of mitochondria can vary depending on the cell's energy requirements. Cells with a higher energy demand, such as neurons will contain a greater number of mitochondria.

Mitochondria consist of two membranes, the outer membrane and the highly folded inner membrane, and two aqueous compartments, the intermembrane space and the matrix (Figure 1.1). The outer membrane contains protein channels referred to as porins that make the membrane permeable to small molecules, such as ions and metabolites with a size of less than 10 kDa (Jap and Walian, 1990; Benz, 1994). The intermembrane space is thought to be essentially contiguous with the cytoplasm in terms of the solutes relevant for mitochondrial function. The inner membrane, on the other hand, is impermeable to most small molecules and ions, allowing only small, uncharged molecules such as water to passively diffuse through it; the inner membrane establishes a barrier between the cytosol and the mitochondrial matrix (Sorgato and Moran, 1993; Ballarin *et al.*, 1996; Amiry-Moghaddam *et al.*, 2005; Calamita *et al.*, 2005). The majority of charged molecules of physiological importance traverse the inner membrane by the means of specific channels or carriers (Ballarin *et al.*, 1996). The inner membrane has a very high protein content and is convoluted and folded into features called cristae, which substantially increase the surface area of the membrane (Palade, 1953). The impermeability of the inner membrane, its high protein content and its topology are critical to the mitochondrion's function in energy generation. The inner membrane is also unique in that it is the exclusive location for the lipid diphosphatidylglycerol, also known as cardiolipin (Daum, 1985). The internal compartment called the matrix space contains the mitochondrial genome and is the site of most of the metabolic processes that occur within the mitochondria. The term "matrix" stems from the observation that this space is very viscous compared to the more aqueous cytoplasm. This is due to the high protein and solute concentrations present within the matrix (Srere, 1987).

1.2.2 *Morphology and Distribution*

Mitochondria may appear as distinct, independent bodies residing within the cytoplasm. However, they can also take on alternative forms. In the budding yeast *Saccharomyces cerevisiae* (Westermann and Neupert, 2000) and in cell culture models (Chen and Chan, 2004), mitochondria form continuous reticular networks. Mitochondria appear as stacks of elongated tubules in the nematode *Caenorhabditis elegans* (Labrousse *et al.*, 1999). The formation of these structures is dynamic, involving a coordinated interplay between fission and fusion events, which are both required for the maintenance of mitochondrial structure and function (Bereiter-Hahn and Voth, 1994; Yaffe, 1999). Modulation of mitochondrial morphology occurs in response to environmental stresses, to changes in energy demands or to increased cell volume. In yeast, a total of 119 genes have been identified as essential for the maintenance of mitochondrial morphology. This illustrates the importance and complexity of mitochondrial maintenance (Altmann and Westermann, 2005).

Mitochondrial fission is not an unexpected phenomenon because mitochondria are not synthesized *de novo*, but rather are formed from pre-existing organelles. Mitochondrial division and proliferation are critical components of cell division because they ensure that daughter cells inherit a sufficient number of mitochondria for viability (Yaffe, 1999). In addition to controlling mitochondrial shape, recent studies have also implicated components of the fission machinery in the regulation of apoptosis (Fannjiang *et al.*, 2004; Jagasia *et al.*, 2005). Yeast proteins that facilitate fission include the mitochondrially associated GTPase Dnm1p (Drp1 in humans) and the outer membrane proteins Caf4p, Fis1p and Mdv1p. Dnm1p is recruited to the mitochondrial surface through interactions with either of the adaptors, Mdv1p or Caf4p, which are tethered to the mitochondrial membrane via Fis1p (Griffin *et al.*, 2005). Fis1p, Mdv1p, and Dnm1p then assemble into active fission complexes on the outer mitochondrial membrane and facilitate mitochondrial division (Bhar *et al.*, 2006; Naylor *et al.*, 2006).

Mitochondrial fusion is also essential; mice defective in mitochondrial fusion die early in development (Chan, 2006). Yeast mitochondrial fusion mutants rapidly lose their mtDNA and become defective for oxidative phosphorylation (Okamoto and Shaw, 2005). The exchange and mixing of protein between fusing mitochondria has also been observed *in vitro* (Nunnari *et al.*, 1997). Mitochondrial fusion events may allow the incorporation of new membrane constituents and proteins into older, damaged organelles. In mammalian cells, two related outer membrane GTPases mitofusin 1 and 2 (Mfn1 and Mfn2) have been identified as components of the fusion machinery (Santel and Fuller, 2001; Santel, 2006). Mitofusins are homologous to the fusion proteins first identified in *Drosophila* (fuzzy onions; fzo) and yeast (Fzo1p) (Hales and Fuller, 1997; Hermann *et* *al.*, 1998). Other proteins such as Mgm1p (OPA1 in humans), a dynamin-like GTPase located in the intermembrane space and Ugo1p, an outer membrane protein that links Fzo1p and Mgm1p GTPases for mitochondrial fusion, are also required (Sesaki *et al.*, 2003; Wong *et al.*, 2003).

Recently, phospholipase D (PLD), a novel mitochondrial outer membrane protein, was shown to be involved in organelle fusion (Choi *et al.*, 2006). PLD-catalyzed production of fusogenic lipids is critical for many types of membrane fusion, such as those mediated by SNAREs (Nakanishi *et al.*, 2006). The presence of a mitochondrial PLD raises the possibility that mitochondrial fusion uses a mechanism common to other cellular fusion events. Defects in morphology proteins affect the balance between fission and fusion, resulting in either excessive mitochondrial interconnectivity (fission defects) or fragmentation (fusion defects).

Mitochondria are dynamic, not only in their morphology but in their movement within the cell. It is becoming increasingly clear that mitochondria do not passively diffuse through the cytosol but rather have a nonrandom distribution within the cell. It is now firmly established that the association with cytoskeletal components greatly influences mitochondrial shape and distribution within the cytoplasm (Bereiter-Hahn and Voth, 1994; Rizzuto *et al.*, 1996). Mitochondrial motility is also essential for the appropriate distribution of organelles between mother and daughter cells during cell division (Simon *et al.*, 1997; Yang *et al.*, 1999).

Evidence has also been emerging that in higher eukaryotes, mitochondria need to be localized strategically to particular subcellular sites to provide energy or to participate in intracellular signaling (Park *et al.*, 2001). In cell culture models, mitochondria have been associated with microfilaments, microtubules and intermediate filaments (Ball and Singer, 1982; Stromer and Bendayan, 1990; Morris and Hollenbeck, 1995). The interaction with the cytoskeleton occurs via docking proteins found on the surface of the mitochondrion. Interaction with the bound molecular motors provides a means for the organelles to move along the cytoskeletal fibers (Deacon *et al.*, 2003; Varadi *et al.*, 2004).

In *S. cerevisiae*, establishment, maintenance and motility of the mitochondrial network is dependent on the actin cytoskeleton (Boldogh *et al.*, 2001; Fehrenbacher *et al.*, 2004). Considerable insight into mitochondrial motility and segregation has been achieved through the isolation of yeast *mdm* (mitochondrial distribution and morphology) mutants (Berger and Yaffe, 1996; Yaffe, 1999). These mutants have aberrant mitochondrial morphologies, compromised intracellular motility and are defective in mitochondrial inheritance during cell division (Sogo and Yaffe, 1994; Berger *et al.*, 1997; Boldogh *et al.*, 2003). The Mdm proteins are localized to both mitochondrial membranes and are thought to mediate the interactions of mitochondria with the cytoskeleton. The proteins Mmm1p, Mdm10p and Mdm12p are integral outer membrane proteins that form a complex localized to punctuate structures in close proximity to mtDNA nucleoids. Deletion of any one of these genes results in the loss of mtDNA or defects in mtDNA nucleoid maintenance (Boldogh *et al.*, 2003). Mmm2p (alternative name for Mdm34p) is located in a separate complex in the outer membrane and is also required for mtDNA nucleoid maintenance (Dimmer *et al.*, 2002; Youngman *et al.*, 2004). The integral inner membrane proteins, Mdm31p and Mdm32p functionally cooperate with the outer membrane machinery to mediate the maintenance of mitochondrial morphology and inheritance of mtDNA (Dimmer *et al.*, 2005). Not surprisingly, cells lacking Mdm31p and Mdm32p also show severe defects in mitochondrial distribution, morphology and mtDNA inheritance.

1.2.3 *Biogenesis*

It is estimated through proteome analysis that approximately 800 proteins reside within the yeast mitochondrion (Sickmann *et al.*, 2003) and approximately 1,500 in humans (Taylor *et al.*, 2003). Although mitochondria possess their own genome and the machinery necessary to carry out protein synthesis, only about 1% of all mitochondrial proteins are encoded by the mtDNA (Wiedemann *et al.*, 2004). The majority of proteins are synthesized in the cytosol as precursor proteins and imported into the mitochondrion (Koehler *et al.*, 1999).

Mitochondrial proteins possess specific targeting and sorting information that directs them to their correct submitochondrial locations. The mitochondrion has evolved an elaborate protein import machinery to facilitate this process. This machinery is responsible for the recognition, translocation, sorting and assembly of imported proteins. Mitochondrial precursor proteins possess either an N-terminal targeting sequence that is characterized as positively charged amphipathic α-helix or they contain multiple internal targeting signals (Schatz and Dobberstein, 1996; Pfanner and Geissler, 2001). The TOM (translocase of the outer membrane) complex resides on the outer membrane where it recognizes incoming precursor proteins via a receptor mediated interaction and facilitates their import into the intermembrane space through a translocation pore (Neupert, 1997; Pfanner and Meijer, 1997; Koehler, 2000). β-barrel outer membrane proteins, such as porin and components of the TOM complex are inserted with the help of the SAM (sorting and assembly machinery of the outer membrane) complex (Wiedemann *et al.*, 2003).

Once the preprotein arrives within the intermembrane space, it can take one of several routes depending on its targeting information. Soluble intermembrane space proteins are folded and assembled with the aid of a number of resident chaperone proteins (Koehler, 2004). Preproteins destined for the mitochondrial matrix depend on the TIM23 (translocase of the inner membrane) complex and its associated import motor. The TIM23 complex identifies proteins with N-terminal extensions and facilitates their membrane potential ($\Delta\psi_m$)-dependent translocation across the inner membrane (Pfanner and Geissler, 2001). Further assistance for protein translocation is provided by the PAM (presequence translocase-associated motor) in the matrix (Neupert and Brunner, 2002). The molecular mechanism of the import motor probably involves both passive trapping and active pulling of the preprotein (Matouschek *et al.*, 2000). Once in the matrix space, preproteins are subsequently processed to their mature forms, folded by chaperones and assembled (Neupert, 1997; Pfanner *et al.*, 1997).

Some proteins contain not only a presequence but also a hydrophobic stop-transfer signal that causes a translocation arrest of the preprotein in the inner membrane. Subsequently, they diffuse laterally into the lipid bilayer of the membrane (Glick *et al.*, 1992). Inner membrane proteins that do not contain an N-terminal presequence but rather possess internal targeting information are recognized by a second translocase, TIM22 (Sirrenberg *et al.*, 1996; Kovermann *et al.*, 2002). These proteins include most multispanning membrane proteins, particularly the members of the metabolite carrier family (Wiedemann *et al.*, 2004). Their transit across the intermembrane space is mediated by the two soluble TIM complexes that exert chaperone-like activities to prevent aggregation of these hydrophobic precursor proteins (Curran *et al.*, 2002; Curran *et al.*, 2002).

The inner membrane also contains additional insertion machinery for proteins that must be exported from the matrix into the inner membrane. These include the integral inner membrane proteins Oxa1p and Mba1p (Bonnefoy, Kermorgant et al. 1994; Preuss, Leonhard et al. 2001; Nargang, Preuss et al. 2002). Substrates for this machinery are encoded by the mtDNA or are one of a few nuclear-encoded proteins directed to the matrix by their presequences, where they follow an export pathway conserved from bacteria (Samuelson *et al.*, 2000).

1.3 Mitochondrial Respiratory Chain

1.3.1 Chemiosmotic Coupling and Oxidative Phosphorylation

The main function of mitochondria is the production of cellular energy by the process known as oxidative phosphorylation (OXPHOS). This process is mediated by five multisubunit protein complexes embedded in the inner membrane, commonly referred to as the mitochondrial respiratory chain (MRC, Figure 1.2) (Hatefi, 1985). The most abundant proteins found in the inner membrane are those involved in OXPHOS. In rat liver mitochondria, they occupy approximately 40% of the inner membrane area (Schwerzmann *et al.*, 1986). In higher eukaryotes, the MRC consists of ~80 structural subunits and ~24 cofactors. The metazoan MRC consists of the following holoenzymes: the NADH-ubiquinone oxidoreductase (complex I), the succinate-ubiquinone oxidoreductase (succinate dehydrogenase, complex II), the ubiquinol-cytochrome *c* oxidoreductases (cytochrome bc_1 complex, complex III), the cytochrome *c* oxidase (complex IV) and the ATP synthase (F_1F_O-ATPase, complex V). The enzymes of the MRC are usually illustrated as distinct complexes residing within the inner membrane; however, an ever increasing amount of evidence suggests the existence of stable interactions between the complexes and the formation of supercomplexes (Schägger and Pfeiffer, 2000; Schägger, 2002). The composition of the supercomplexes varies with the detergent extraction conditions and between organisms (Stroh *et al.*, 2004). Supercomplex formation is proposed to facilitate substrate channeling, improve catalytic efficiency and stabilize the individual complexes (Bianchi *et al.*, 2004).

The MRC generates ATP through chemiosmotic coupling (Mitchell and Moyle, 1967; Mitchell, 1979), which is also used in other electron transport systems, such as the thylakoid membrane of chloroplast and the plasma membrane of respiring and photosynthetic prokaryotes (Packer, 1974). The MRC shuttles electrons extracted from the oxidation of molecules such as fatty acids and glucose along a series of cofactors of increasing reduction potential. In mitochondria, the final electron acceptor of this process is molecular oxygen, which is reduced to water. Coupled to electron transport, protons are pumped across the inner membrane from the matrix into the intermembrane space by complexes I, III and IV. Due to the impermeability of the inner membrane, a proton gradient is established; the redox energy of respiration is stored in the form of a transmembrane electrochemical gradient ($\Delta\mu_{H+}$), also referred to as the proton motive force (Δp). Δp consists of both a pH difference (ΔpH) due to the different concentrations of protons across the inner membrane and an electrical or membrane potential ($\Delta\psi_m$). Δp can be expressed as the sum of the ΔpH and $\Delta\psi_m$ contributions in the following equation (Mitchell, 1979; Mitchell and Moyle, 1979):

$$\boldsymbol{\Delta p = (-2.303RT\Delta pH)/nF + \Delta\psi_m}$$

where R is the universal gas constant of 8.315 J K^{-1} mol^{-1}

T is the temperature in Kelvin

n is the moles of protons

F is the Faraday constant of 96 494 J V^{-1} mol^{-1}

The formation of a Δp by energy transducing membranes is the central feature of chemiosmotic coupling. The ATP synthase (complex V) couples energy from the proton motive force during the flow of protons back through the inner membrane to the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). The proton gradient also provides energy for thermogenesis in multicellular organisms (Diehl and

Hoek, 1999) and is necessary for protein import across the inner membrane (Neupert, 1997).

1.3.2 NADH-Ubiquinone Oxidoreductase (Complex I)

Complex I is the largest and most structurally complicated member of the MRC. It acts as the main entry point for electrons entering the MRC (Walker, 1992). Complex I functions to oxidize reduced nicotinamide adenine dinucleotide (NADH) to NAD^+, transferring two electrons through a series of cofactors to the lipid soluble electron carrier ubiquinone (coenzyme Q). Flavin mononucleotide (FMN) at the active site accepts two electrons simultaneously (as a hydride ion) from NADH and transfers them one at a time to a chain of iron-sulfur (Fe-S) clusters, which are one-electron carriers. The last Fe-S cluster reduces ubiquinone to ubiquinol in two one-electron steps. Ubiquinol shuttles the electrons to complex III, where it is re-oxidized. The transfer of the electrons through complex I is coupled to the translocation of four protons across the inner mitochondrial membrane (Brown and Brand, 1988). The smallest version of complex I exists in *E. coli*, where it comprises 14 subunits, FMN and up to nine Fe-S clusters (Leif *et al.*, 1993; Yagi *et al.*, 1998). The bovine complex I, the best characterized metazoan enzyme, consists of 45 subunits, seven of which are encoded by the mtDNA, FMN and eight Fe-S clusters (Carroll *et al.*, 2006). It has an estimated molecular weight of 980 kDa, assuming a single copy of each subunit is incorporated into the holoenzyme. Fungal species such as *Yarrowia lipolytica* (Djafarzadeh *et al.*, 2000) and *Neurospora crassa* (Videira, 1998; Videira and Duarte, 2002) contain Complex I but this enzyme is absent in *S. cerevisiae*. Instead *S. cerevisiae* possesses a single subunit enzyme called Ndi1p, which transfers electrons from NADH to ubiquinone but in contrast to the multisubunit respiratory complex I holoenzymes, does not pump protons (Marres *et al.*, 1991; De Vries *et al.*, 1992). Ndi1p is also insensitive to the classical inhibitors of complex I, such as rotenone or piericidin (de Vries and Grivell, 1988).

Mitochondrial and bacterial complex I have a characteristic L-shaped structure, with a hydrophobic arm embedded in the membrane and a hydrophilic, peripheral arm protruding into the mitochondrial matrix or the bacterial cytoplasm (Guénebaut *et al.*, 1997; Grigorieff, 1998; Peng *et al.*, 2003). The hydrophilic domain of complex I contains the NADH-binding site, the FMN and the Fe-S clusters (Ohnishi, 1998; Yagi and Matsuno-Yagi, 2003), whereas the proton-pumping machinery is thought to be in the membrane arm. Recently, the crystal structure of the hydrophilic domain (peripheral arm) of the *Thermus thermophilus* complex I was reported (Sazanov and Hinchliffe, 2006). This subcomplex consists of eight hydrophilic subunits (Nqo1 to 6, Nqo9 and Nqo15) and contains all the redox centers of the enzyme, including nine Fe-S clusters. A linear pathway containing the FMN and seven Fe-S centers (designated N3, N1b, N4, N5, N6a, N6b and N2) leads to a proposed quinone binding site. A distal Fe-S cluster (N1a), which is adjacent to the FMN molecule at the beginning of the chain, is proposed to function in the temporary storage of electrons, possibly to minimize ROS production during turnover of complex I. An additional Fe-S center, cluster N7 in subunit Nqo3 is too far from the other clusters to accept electrons efficiently and is not part of the proposed electron transfer pathway (Hinchliffe and Sazanov, 2005; Sazanov and Hinchliffe, 2006).

Despite the recent advances in understanding the inner workings of this extremely complex enzyme, little is still known about the mechanism of proton translocation for complex I. Two main models for coupling electron transfer to proton translocation have been proposed: direct (redox-driven, e.g., Q-cycle variations) or indirect (through conformational changes) (Belogrudov and Hatefi, 1994; Friedrich, 2001; Mamedova *et al.*, 2004). Recent structural information hints that both may be involved, however more structural and functional information is required to definitively evaluate these models.

1.3.3 Ubiquinol-Cytochrome c Oxidoreductase (Complex III)

Complex III is the best understood of the MRC complexes. It is a member of the *bc*-type superfamily, which includes the cytochrome *bf* complexes found in chloroplasts, algae and some gram-positive bacteria (Trumpower, 1990; Trumpower and Gennis, 1994; Berry *et al.*, 2000). Complex III couples the transfer of electrons from ubiquinol to cytochrome *c* to the translocation of protons across in the inner membrane. It is present as a symmetrical dimer in the inner mitochondrial membrane (Iwata *et al.*, 1998; Hunte *et al.*, 2000). The dimer not only has a structural role, but is also essential to the mechanism of energy conservation (Covian and Trumpower, 2005).

The metazoan holoenzyme is comprised of 11 subunits, while the *S. cerevisiae* cytochrome bc_1 complex is composed of 10 subunits (Tzagoloff, 1995). The cytochrome *b*, cytochrome c_1 and the Rieske iron-sulfur protein (ISP) subunits contain all the redox-active centers and constitute a highly conserved catalytic core of the holoenzyme (Saraste, 1999). In some bacterial bc_1 complexes, only the core subunits are present (Trumpower, 1990). Cytochrome *b*, the only subunit of complex III encoded by the mtDNA, possesses two *b*-type hemes, designated b_L and b_H for the low and high potential hemes, respectively. In addition to these hemes, the subunit has two quinone binding sites, designated Q_i and Q_o sites for their locations on the inner and outer surfaces of the inner membrane, respectively. The ISP contains a high potential [2Fe-2S] cluster and the cytochrome c_1 subunit possesses a *c*-type heme that acts as the electron donor to cytochrome *c*. Cytochrome *c* is a soluble, heme-containing protein of the intermembrane space that mediates electron transfer between complexes III and IV (Lange and Hunte, 2002). The non-core subunits are termed supernumerary subunits and are not essential for catalysis, although some have important roles in the structural organization of the bc_1 complex (Zara *et al.*, 2004). The supernumerary core protein 1 and core protein 2 subunits are members of the mitochondrial processing peptidase (MPP) family of proteins, making the bc_1 complex a bifunctional complex also involved in mitochondrial protein import (Braun *et al.*, 1992).

Electron transfer within complex III is much more complicated than in other complexes, which are known to or thought to have linear pathways. The movement of electrons involves a branched transfer pathway known as the Q-cycle (Brandt and Trumpower, 1994; Zhang *et al.*, 1998; Crofts, 2004). One electron is passed from ubiquinol at the Q_o site to the Rieske iron-sulfur cluster and cytochrome c_1; this is known as the high-potential chain. The second electron is transferred first to hemes b_L and b_H and to the Q_i site, where it reduces ubiquinone to an ubisemiquinone intermediate. In a second round of electron transfer, the ubisemiquinone is fully reduced to ubiquinol. The net effect of each reaction cycle is the oxidation of two molecules of ubiquinol producing two reduced cytochrome *c* molecules and the regeneration of one molecule of ubiquinol. Two protons from the matrix are consumed in the regeneration of the ubiquinol molecule and four protons are released into the intermembrane space from the oxidation of the two ubiquinol molecules that are bound at the Q_o site.

1.3.4 Cytochrome c Oxidase (Complex IV)

The final step in respiration is catalyzed by complex IV and this involves the transfer of electrons from cytochrome *c* to oxygen resulting in the production of H_2O (Capaldi, 1990; Malatesta *et al.*, 1995; Michel *et al.*, 1998). This electrogenic reaction consumes four hydrogen ions from the matrix to produce water and is coupled to the translocation of four more protons across the inner membrane. Cytochrome *c* oxidases belong to a superfamily of heme/copper-containing terminal oxidases that includes the bacterial cytochrome *bo* complex (Capaldi, 1990; Saraste, 1990). The composition of the mitochondrial holoenzyme ranges from 7 to 13 non-equivalent subunits with the three largest subunits, COX I - III being encoded by the mitochondrial genome. The metazoan complex IV contains 13 subunits (Tsukihara *et al.*, 1996), whereas the *S. cerevisiae* holoenzyme contains only 11 (Taanman and Capaldi, 1992). Subunits I - III are highly conserved through evolution and contain all the redox-active metal centers involved in electron transfer. These three subunits constitute the minimal catalytic core; simpler bacterial enzymes such as that from *Paracoccus denitrificans* can oxidize cytochrome *c* and reduce oxygen to water with the three core subunits and a small non-conserved subunit IV (Iwata *et al.*, 1995; Michel *et al.*, 1998).

Subunit I possesses a copper ion, Cu_B and two chemically identical though spectrally distinct heme *a* species: a six-coordinate low spin heme *a* and a five coordinate high spin heme a_3. The Cu_B and heme a_3 form a copper-heme binuclear center, which is the site for oxygen binding and reduction (Babcock and Wikstrom, 1992). Subunit II contains a copper-copper binuclear center referred to as Cu_A, which serves as the entry point for electrons arriving from cytochrome *c* (Capaldi, 1990). Heme *a* sits between the two binuclear centers and mediates the transfer of electrons from Cu_A to the heme a_3-Cu_B redox center. The function of subunit III remains unknown, although it has been suggested that it forms the entrance to an oxygen channel leading to the active site (Riistama *et al.*, 1996). The nuclear encoded subunits are thought to facilitate the assembly of the holoenzyme, regulate its catalytic activity and protect the catalytic core from ROS damage (Capaldi, 1990; Fontanesi *et al.*, 2006). Seven of the subunits (IV, VIa, VIc, VIIa, VIIb, VIIc, and VIII) are small, integral membrane proteins, while subunits Va, Vb and VIb are peripheral membrane proteins (Tsukihara *et al.*, 1996). Subunit Vb (yeast Cox4p), a non catalytic subunit possesses a coordinated zinc ion that is essential for stability of the complex (Coyne *et al.*, 2007).

1.3.5 ATP Synthase (Complex V)

Complex V harnesses the energy stored in the proton motive force to catalyze the synthesis of ATP from ADP and P_i (Fillingame, 1999; Yoshida *et al.*, 2001). Structurally, the enzyme resembles a turbine, where the flow of protons drives the rotation of the head portion of the enzyme to power the synthesis of ATP (Sambongi *et al.*, 1999; Stock *et al.*, 1999). Complex V consists of two major structural parts, a globular catalytic head domain that is referred to as F_1 and the integral membrane portion known as F_O (Seelert *et al.*, 2000; Rubinstein *et al.*, 2003). The *E. coli* enzyme is the simplest, containing only 8 subunits while the yeast ATP synthase is the most complex known and is made of 20 different types of subunits, with three being encoded by the mtDNA (Fillingame and Divall, 1999; Velours and Arselin, 2000). The mammalian enzyme contains at least 16 different subunits, with two subunits being derived from the mtDNA (Collinson *et al.*, 1994).

The F_1 domain is an assembly of five different proteins α, β, γ, δ and ε with a stoichiometry of 3:3:1:1:1. In the F_1 domain, the three α-subunits and the three β-subunits are arranged alternately around a central α-helical coiled-coil in the γ-subunit. The three nucleotide binding sites are formed at the interface between the α and *β* subunits (Abrahams *et al.*, 1994; Menz *et al.*, 2001). They exist in either the tight (where catalysis occurs), loose (where substrates are bound) or open (with low affinity for nucleotide) conformation at any given point in during the binding change mechanism of catalysis but interconvert sequentially between these different conformations as catalysis proceeds (Boyer, 1993; Boyer, 1997). The γ, δ and ε subunits form a central stalk that links the F_1 domain to F_O. It is the rotation of the central stalk along its long axis within the αβ assembly, altering the structure of the β subunit that drives ATP synthesis (Gibbons *et al.*, 2000). The foot of the central stalk interacts with the F_O portion of the enzyme, which consists of a ring of 10-14 *c* subunits depending on the species examined (Fillingame *et al.*, 1998; Böttcher and Graber, 2000). Rotation of the *c*-ring and of the central stalk ensemble is driven by the passage of protons through a channel that is formed between the *c* ring and subunit a, encoded by the *ATP6* gene found on the mtDNA (Stock *et al.*, 2000; Fillingame and Dmitriev, 2002).

In addition to the two major domains of the ATP synthase, more recently, a peripheral stalk domain has been identified. This domain consists of eight non-equivalent subunits: OSCP (oligomycin sensitivity-conferring protein), b, d, e, f, g, A6L and F_6. The *ATP8* gene of the mtDNA encodes the A6L protein. The peripheral stalk domain contains a single copy of the b, d, and F_6 subunits while the stoichiometries of the other subunits are still unknown (Collinson *et al.*, 1996; Walker and Dickson, 2006). The F_1 and the F_O

domains are linked by this peripheral stalk and it functions as a stator to hold the peripheral membrane domain and the $(\alpha\beta)_3$ subcomplex in place relative to the central stalk and rotating *c* ring (Dickson *et al.*, 2006). There is also a regulatory subunit called inhibitor factor 1 (IF_1), which inhibits F_1 activity by docking to β subunits in two neighboring ATPase complexes. IF_1 functions under conditions when Δp has collapsed, such as during ischemia, preventing the hydrolytic action of the ATP synthase (Gledhill and Walker, 2006).

1.4 Succinate-Ubiquinone Oxidoreducatase (Complex II, SDH)

1.4.1 Structural and Functional Properties of SDH

Complex II is the simplest of the MRC enzymes with the eukaryotic version containing only four subunits. Known more commonly as succinate dehydrogenase (SDH), this enzyme catalyzes the oxidation of succinate to fumarate coupled to the reduction of ubiquinone to ubiquinol (Ackrell *et al.*, 1992; Hägerhäll, 1997). This enzyme is unique among the members of the MRC since it does not contribute to the generation of the membrane potential (Cecchini, 2003). SDH is also involved in the Krebs (tricarboxylic acid) cycle, creating a link between these two essential metabolic processes (Figure 1.3). The *S. cerevisiae* SDH, our model of the eukaryotic SDH, is a member of the succinate-ubiquinone oxidoreductase (SQO) superfamily of proteins, which is characterized by a catalytic heterodimer containing covalent FAD and three iron-sulfur clusters (Singer *et al.*, 1957; Singer *et al.*, 1965; Lemire and Oyedotun, 2002). Also members of the superfamily are the bacterial fumarate reductases (FRD), which catalyze the reverse reaction of SDH; the reduction of fumarate to succinate coupled with the oxidation of quinol (Cecchini, 2003). FRD is normally expressed under anaerobic conditions where as SDH expression occurs under aerobic conditions. Both enzymes can catalyze the same reactions *in vivo* and *in vitro*, highlighting their high degree of sequence, structural and functional similarities (Maklashina *et al.*, 1998; Cecchini, 2003). SQO members can be classified into five types (A-E) according to the number of heme groups and hydrophobic subunits. The mitochondrial SDH is a type C enzyme; it contains one heme group and two transmembrane proteins (Table 1.1) (Lemos *et al.*, 2002).

The elucidation of the tetrameric structure of SDH is based on a combination of genetic, biochemical and structural analyses from various model systems. The recent determination of the *E. coli*, avian and porcine SDH X-ray crystal structures have provided excellent structural information about the enzyme and have confirmed structural models of the enzyme based on previous biochemical and biophysical analyses (Yankovskaya *et al.*, 2003; Huang *et al.*, 2005; Sun *et al.*, 2005). The structures of the *E. coli* and *W. succinogenes* FRDs have also been reported (Iverson *et al.*, 1999; Lancaster *et al.*, 1999). The abundance of structural information has provided a framework for understanding the mechanistic details of the enzyme. A model of the yeast SDH has been calculated; the yeast enzyme closely resembles its mammalian counterpart in its catalytic and structural properties and in its flavin and iron-sulfur cofactor content (Robinson and Lemire, 1995; Robinson and Lemire, 1996; Oyedotun and Lemire, 2004). The overall architecture of yeast SDH consists of a globular catalytic domain consisting of the Sdh1p and Sdh2p subunits attached to the membrane anchor domain consisting of the Sdh3p and Sdh4p subunits (Figure 1.4). The yeast SDH has an apparent molecular mass of approximately 150 kDa as determined by blue native gel electrophoresis; this differs slightly from the predicted mass of 132 kDa (Schägger and von Jagow, 1991).

Although SDH functions as an individual holoenzyme, there is evidence to suggest it is organized as a component of a supercomplex in the yeast mitochondrial respiratory chain. Kinetic studies suggest that the yeast MRC functions as a single unit (Boumans *et al.*, 1998). Mutations to subunit 8 of cytochrome bc_1 complex affect SDH activity, suggesting a physical interaction between the two enzymes (Bruel *et al.*, 1996; Boumans *et al.*, 1998). The formation of this supercomplex may enhance the cell's ability to adapt to rapidly fluctuating availability of different substrates (Boumans *et al.*, 1998). In addition to its presence in supercomplexes, SDH may also form trimers in the membrane as suggested by the organization of the enzyme in crystals of the *E. coli* SDH. SDH monomers were very tightly packed with a contact surface of 1,242 Å^2, suggesting that this trimer association is physiological (Yankovskaya *et al.*, 2003). However, this was not observed for the mammalian enzyme and may represent a feature only seen in prokaryotes (Sun *et al.*, 2005).

While SDH is known mainly for its roles in the MRC and the Krebs cycle, it has been shown to be involved in other processes as well. SDH is functionally and structurally part of the mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$). This channel is a key player in the process of ischemic preconditioning, an endogenous autoprotective mechanism activated to prevent cellular injury during future ischemic episodes (Gross and Fryer, 1999). Evidence for the involvement of SDH in mitoK$_{ATP}$ channel function includes the observation that SDH inhibitors block channel activity and that SDH can be co-immunoprecipitated with the channel (Ardehali *et al.*, 2004). The channel appears to be multiprotein complex of the mitochondrial inner membrane that also contains the ATP-binding cassette protein 1, the phosphate carrier, the adenine nucleotide translocator and the ATP synthase. It has been proposed that SDH regulates mitoK$_{ATP}$ function through a physical interaction with the channel rather than through its role in oxidative phosphorylation (Ardehali *et al.*, 2004).

1.4.2 Molecular Biology of SDH

Among the members of the MRC, SDH is the only one with all of its subunits encoded by the nuclear genome. The other four complexes each contain one or more mtDNA encoded subunits. In most organisms, such as humans and *E. coli*, the genes are referred to as *SDHA-D*, while in *S. cerevisiae* the genes are referred to as *SDH1-4*. In yeast, mutations in any one of the four unlinked SDH genes leads to the loss of SDH function and the ability to grow by respiration (Lemire and Oyedotun, 2002). In eubacteria, the SDH genes are usually found in an operon, *sdhCDAB* in *E. coli* and *sdhABCD* for most archaeal complexes (Schafer *et al.*, 2002; Cecchini, 2003). In all cases, the polypeptide containing the dicarboxylate binding site and the flavin cofactor is encoded by the *SDHA* or *SDH1* genes, and the iron-sulfur containing subunit is encoded by the *SDHB* or *SDH2* genes. The *SDH3/SDHC* and *SDH4/SDHD* genes encode the hydrophobic anchor subunits that reside in the either the bacterial plasma membrane or mitochondrial inner membrane (Cecchini, 2003).

Although the majority of organisms possess the canonical four subunit holoenzyme encoded by the nuclear genome, exceptions to this rule exist. Organisms such as the red algae *Porphyra purpurea* and heterotrophic zooflagellate *Reclinomonas americana* possess mitochondrially encoded genes for *SDHB-D* (Burger *et al.*, 1996). These observations support the notion that the evolution of citric acid cycle proteins originated from within the α-proteobacterial branch of eubacteria (Burger *et al.*, 1996; Schnarrenberger and Martin, 2002). Other exceptions include the *Wolinella succinogenes* FRD and the *Bacillus subtilis* SDH, which contain fused *C* and *D* genes resulting in three subunit enzymes (Lauterbach *et al.*, 1990; Hederstedt, 2002).

1.4.3 Catalytic Dimer

In yeast, the soluble catalytic domain consists of a heterodimer of Sdh1p and Sdh2p subunits. Sdh1p is known as the flavoprotein (Fp) subunit because of its covalently bound FAD prosthetic group (Singer *et al.*, 1957). Sdh1p also harbours the dicarboxylate substrate binding site. Sdh1p is a hydrophilic 65-79 kDa polypeptide exposed to the matrix side of the mitochondrial inner membrane or to the prokaryotic cytoplasm (Hägerhäll, 1997). This subunit exhibits a high degree of conservation throughout evolution; with the yeast Sdh1p having 53% and 68% sequence identity with the *E. coli* and porcine subunits, respectively. Deletion of the *SDH1* gene results in respiration deficiency, the loss of SDH activity and the absence of trichloroacetic acid precipitable flavin (Robinson *et al.*, 1991; Robinson and Lemire, 1992) . In yeast, Sdh1p is the predominant covalent flavoprotein, a feature that is exploited in our studies of the enzyme (Lemire and Oyedotun, 2002).

The FAD is the redox active center and is essential for catalysis (Robinson *et al.*, 1994; Maklashina *et al.*, 2006). It is located adjacent to the substrate binding site and is the first electron acceptor during succinate oxidation (Figure 1.5). The FAD is covalently attached via an 8(α)-N(3) histidyl linkage to His-90 (Singer *et al.*, 1957; Walker and Singer, 1970). The covalent attachment of FAD in SDH and FRD has been an area of particular interest because soluble FRD homologs from various species contain a noncovalently bound FAD (Arikawa *et al.*, 1998; Besteiro *et al.*, 2002). It has been suggested that covalent attachment may occur to prevent loss of the flavin cofactor or to prevent competing side reactions during catalysis (Decker, 1993; Mewies *et al.*, 1998). More recent evidence suggests that the covalent linkage increases the redox potential of the FAD, a requirement for succinate oxidation (Blaut *et al.*, 1989). The increased redox potential is a consequence of both the covalent linkage and of the protein environment around the FAD (Efimov *et al.*, 2001) . The yeast enzyme harbouring a His-90 mutation in Sdh1p contains noncovalent but tightly bound FAD; this mutant is unable to oxidize succinate (Robinson *et al.*, 1994).

Covalent FAD attachment is believed to be an autocatalytic event. As shown for the yeast SDH, the processed and folded Sdh1p subunit is required for covalent flavinylation (Robinson and Lemire, 1996). C-terminal truncations at sites far removed from the FAD binding site prevent FAD attachment. The mitochondrial chaperone Hsp60 enhances the flavinylation reaction, although it is not absolutely required (Robinson and Lemire, 1996). Flavinylation can also be stimulated by Sdh2p and tricarboxylic acid intermediates such as succinate, malate or fumarate, consistent with an autocatalytic reaction mechanism (Robinson and Lemire, 1996). The *B. subtilis* SDH does not undergo covalent flavin attachment during heterologous expression in *E. coli* (Hederstedt *et al.*, 1987), an observation that has been used to argue against an autocatalytic mechanism. However, it is now believed that FAD attachment for most covalent flavoproteins is autocatalytic (Mewies *et al.*, 1998).

The active site of SDH is able to bind a number of dicarboxylate substrates. These include succinate, fumarate, L-chlorosuccinate and malate (Ackrell *et al.*, 1992; Hägerhäll, 1997). The substrate affinity at the active site is dependent on the redox state of the enzyme. SDH has a higher affinity for dicarboxylates in the oxidized state (Kotlyar and Vinogradov, 1984), suggesting that the redox state of the enzyme governs the conformation of the active site and the binding of substrate. SDH is inhibited by malonate, a molecule whose structure closely resembles succinate. It is also inhibited by oxaloacetate, a metabolite of the tricarboxylic acid cycle (Ackrell *et al.*, 1992). Both are potent competitive inhibitors that bind to the active site with affinities in the micromolar range. D- and L-malate are poor substrates for SDH because they are oxidized to enol-oxaloacetate, which inhibits the enzyme (Panchenko and Vinogradov, 1991). Largely based on work with FRD, a catalytic mechanism for succinate oxidation has been proposed (Lancaster *et al.*, 1999; Doherty *et al.*, 2000; Pankhurst *et al.*, 2002; Cecchini, 2003). Pairs of histidine and arginine residues are thought to participate in catalysis (Schröder *et al.*, 1991). These residues line one side of the substrate binding pocket with the isoalloxazine ring of the FAD lining the other side. A histidine and an arginine pair on the opposing side of the substrate binding site align the substrate at the active site via electrostatic interactions. A second arginine and histidine pair are positioned over the carbon chain of the substrate and either residue may participate in protonation/deprotonation of the substrate. Histidine is more likely to participate in catalysis by functioning as a general acid-base catalyst (Vik and Hatefi, 1981).

The iron-sulfur subunit (Ip) forms the catalytic dimer with the flavoprotein subunit. Referred to as Sdh2p in yeast and SdhB in other organisms, this subunit is highly conserved throughout evolution. It has a mass of 27-31 kDa and contains three iron-sulfur clusters named center 1, a $[2Fe\text{-}2S]^{2+,1+}$ cluster, center 2, a $[4Fe\text{-}4S]^{2+,1+}$ cluster and center 3, a $[3Fe\text{-}4S]^{1+,0}$ cluster (Ackrell *et al.*, 1992; Hägerhäll, 1997). Three iron-sulfur centers are found in the majority of SQO family members, although with differing redox properties that reflect the physiological function of each particular enzyme (Ackrell *et al.*, 1992).

In the yeast Sdh2p, all three clusters are ligated to the polypeptide via conserved cysteine motifs (Lemire and Oyedotun, 2002). All three iron-sulfur centers are expected to participate in electron transfer because the edge-to-edge distances between adjacent redox centers in the catalytic domain are within 14 Å, the physiologically relevant distance for electron transfer (Figure 1.5) (Page *et al.*, 1999). Center 1 is the immediate electron acceptor from the FAD. From there, the electrons flow through centers 2 and 3 before entering the membrane anchor domain (Yankovskaya *et al.*, 2003; Sun *et al.*, 2005). The low midpoint potential of the [4Fe-4S] cluster does not appear to present a significant thermodynamic barrier to electron flow (Ohnishi *et al.*, 2000). It has been suggested that center 3 may also have a structural role in the docking of the catalytic and membrane domains (Manodori *et al.*, 1992).

1.4.4 Membrane Dimer

In contrast to the catalytic dimer subunits, there is a large diversity in sequence, structure, subunit and cofactor composition observed in the anchor polypeptides (Hägerhäll and Hederstedt, 1996; Hägerhäll, 1997). The membrane domain plays a major role in determining the unique properties of each enzyme in the SQO family because it interacts with a variety of quinone and quinol species (Table 1.1) (Hägerhäll, 1997; Tielens and VanHellemond, 1998). The membrane subunits help define whether an enzyme is an SDH or FRD and establishing the preferred direction of electron flow (Lemire and Oyedotun, 2002).

In the eukaryotic SDH, the membrane anchor domain is comprised of a heterodimer of two smaller hydrophobic subunits, Sdh3p/SdhC and Sdh4p/SdhD. These subunits are also referred to as cytochrome *b* large (CybL) and cytochrome *b* small (CybS) subunits, respectively (Lemos *et al.*, 2002). They vary in size from 13 to 18 kDa for SdhC and from 11 to 16 kDa for SdhD. Both of the yeast subunits have an approximate molecular weight of 16 kDa (Schägger and von Jagow, 1991; Bullis and

Lemire, 1994; Lemire and Oyedotun, 2002). Each subunit is comprised of three transmembrane helices and contributes two helices to an anti-parallel four-helix bundle that is the core of the hydrophobic domain. Both subunits contribute residues that interact with cofactors found in the membrane anchor domain (Yankovskaya *et al.*, 2003; Huang *et al.*, 2005; Sun *et al.*, 2005). Three subunit members of the SQO family have their membrane subunits fused into one larger hydrophobic polypeptide (Cecchini, 2003). The four-helix bundle core structure remains intact; the third helix of Sdh3p/SdhC subunit is absent (Hägerhäll, 1997; Hederstedt, 1999).

In the center of the four helix bundle of the mitochondrial SDH resides a single *b*-type heme. The heme is common to SDHs in most organisms. In contrast, the *B. subtilis* SDH contains two hemes and the number of hemes in FRDs ranges from zero to two (Hägerhäll, 1997). The variety of heme contents has raised questions as to its role. It has been proposed that the heme is important for catalysis and for structural stability of the enzyme (Hägerhäll, 1997; Hederstedt, 2002). The protoheme IX molecule is normally coordinated within the membrane anchor domain by the imidazole side chains of two histidine residues; in yeast, one histidine in Sdh3p and one cysteine in Sdh4p are the ligands (Hägerhäll and Hederstedt, 1996; Hägerhäll, 1997; Oyedotun *et al.*, 2004). The heme propionate groups interact with several surrounding polar residues and a few additional hydrophobic interactions between the porphyrin group and the four helices of the helix bundle further stabilize the bound heme (Yankovskaya *et al.*, 2003; Huang *et al.*, 2005; Sun *et al.*, 2005). It should be noted the interactions between the heme and the surrounding four transmembrane helices are not extensive. The stabilization of the heme within the membrane domain occurs mainly through its interaction with the axial ligands.

This is highlighted by the observation that mutation of both axial ligands in the yeast SDH results in an assembled but heme-free enzyme (Oyedotun *et al.*, 2007). Molecular dynamics simulations on the mutant SDH suggest that heme retention in the pocket is not feasible in the absence of both axial ligands (Oyedotun *et al.*, 2007). Heme *b* is retained at significant levels, although with altered spectral properties when either of the axial ligands of the yeast enzyme is mutated (Oyedotun *et al.*, 2004). A similar result was observed in the *E. coli* enzyme when either of the axial ligands was mutated (Maklashina *et al.*, 2001). This indicates that either ligand can provide sufficient coordination for the heme to be retained within the anchor domain.

Current evidence does suggest that the heme is important for the structural stability of the enzyme. The *E. coli* SDH containing mutations to either axial ligand was found to be less thermostable and more sensitive to aeration (Maklashina *et al.*, 2001). The heme was proposed to play a significant structural role in the *E. coli* SDH given its interactions with residues in the SdhB, SdhC and SdhD subunits (Yankovskaya *et al.*, 2003). For the yeast SDH, mutation of either or both of the axial ligands resulted in decreased amounts of assembled enzyme (Oyedotun *et al.*, 2004; Oyedotun *et al.*, 2007). This has been attributed to a decreased stability of the enzyme and was confirmed by molecular dynamics simulations, which showed that the absence of the heme destabilizes the enzyme. The destabilizing effect was localized to the heme-binding region and does not cause the enzyme to completely unfold (Oyedotun *et al.*, 2007). These results indicate that although the heme does provide some structural stability, its presence is not obligatory for the maintenance of the correct tertiary and quaternary structures of the enzyme.

The role of the heme as a redox cofactor is also controversial (Hägerhäll, 1997). For some members of the SQO family, the heme clearly functions in electron transfer. These include the *B. subtilis* SDH and the *W. succinogenes* FRD. In the *B. subtilis* SDH, the high potential heme can be reduced by succinate at the same rate as enzyme turnover. Similarly, for the *W. succinogenes* FRD, high potential heme can be reduced by menaquinol analogues and reoxidized by fumarate at the same rate as enzyme turnover (Unden *et al.*, 1984; Matsson *et al.*, 2000). The *W. succinogenes* FRD contains an additional low potential heme and both are likely to participate in electron transfer since they are separated by a distance of 4.2 Å (Lancaster *et al.*, 1999). In enzymes that contain a single heme, its involvement in electron transfer is less clear. While the heme in *E. coli* SDH is fully reducible by succinate, the *Ascaris suum* enzyme is only partially reduced and the heme in the bovine SDH is not reducible by succinate (Yu and Yu, 1982; Takamiya *et al.*, 1986; Kita *et al.*, 1989; Takamiya *et al.*, 1990). The midpoint potentials of the hemes vary considerably and the heme redox potential of the bovine SDH (-185 mV) is too low for reduction by succinate (Yu *et al.*, 1987). Furthermore, in the eukaryotic enzyme, the redox potential of the heme is much lower than that of the [3Fe-S] cluster (+60 mV) suggesting that ubiquinone reduction (+113 mV) is more thermodynamically favourable (Figure 1.5). It was also recently demonstrated in the *E. coli* enzyme that quinone reduction occurs prior to any electron transfer to heme *b* (Tran *et al.*, 2006). These observations indicate that electrons flowing from the [3Fe-4S] cluster are transferred to ubiquinone without the involvement of the heme *b* and that it would not normally participate in electron transfer under physiological conditions. Consistent with these results is the recent finding that the heme does not appear to be essential for quinone reduction (Oyedotun *et al.*, 2007). A significant amount of quinone reductase activity (~70% of wild-type) was observed in the heme-free yeast SDH. However, this enzyme does not function optimally, with the catalytic efficiency decreased by an order of magnitude compared to the wild-type. This indicates an altered affinity of the enzyme for quinone. These findings were not attributed to a role for the heme in catalysis but rather to structural perturbations in the membrane anchor domain caused by the absence of heme. Structural changes introduced by the loss of heme appear to be propagated to the Q_P site, indirectly affecting the affinity of the enzyme for quinone (Oyedotun *et al.*, 2007).

Although the evidence suggests that heme is not involved in electron transfer during succinate oxidation, it may function in fumarate reduction or reverse electron transfer. Reduced heme *b* in the *E. coli* SDH can be rapidly oxidized by fumarate at the same rate as the turnover of enzyme (Cecchini, 2003). Similarly, heme *b* in both the yeast and bovine SDH, once reduced, is also rapidly oxidized in the presence of fumarate (Hatefi and Galante, 1980; Yu *et al.*, 1987; Oyedotun and Lemire, 1999). Based on this culmination of evidence, it has been suggested that heme *b* does not participate in electron transfer from succinate oxidation but may become part of the electron transfer pathway in reverse direction due its close proximity to the quinone and [3Fe-4S] cluster (Cecchini *et al.*, 2002).

The question remains as to why the enzyme would incorporate a heme molecule into its structure. One suggestion is that the heme acts as an electron sink to draw electrons towards the quinone binding site and away from the FAD and thus preventing ROS formation (Yankovskaya *et al.*, 2003). When comparing the *E. coli* SDH and the *E.*

coli FRD, the most obvious structural difference is that FRD lacks a heme moiety in its membrane anchor domain (Cecchini *et al.*, 2002). FRD is normally expressed under anaerobic conditions, but when expressed aerobically, succinate oxidation by FRD generates a significant amount of ROS derived from the FAD (Messner and Imlay, 2002). SDH on the other hand is not a major source of ROS under normal physiological conditions (Raha and Robinson, 2001; Messner and Imlay, 2002). Evolutionary pressure from an oxygen rich environment may have caused aerobic organisms to preferentially choose SDH over FRD to limit the production of damaging ROS (Yankovskaya *et al.*, 2003). Another proposed function for heme *b* is that it could act as a capacitor/voltage regulator during periods of high electron flux (Oyedotun *et al.*, 2007). As demonstrated in the *E. coli* SDH, heme *b* is only fully reduced once four electrons have been introduced into the protein (Anderson *et al.*, 2005). Therefore, electrons could be temporarily stored on heme *b* before being safely transferred to the bound ubiquinone molecule.

The heme may have additional functions in metazoan physiology. It has been proposed to play a role in oxygen sensing and the cellular response to hypoxic conditions (Baysal *et al.*, 2000). A His102Leu mutation in the human *SDHD* gene causes paraganglioma, a benign, vascularized tumor in the head and neck region. This histidine residue is one of the axial ligands to the heme *b* (Sun *et al.*, 2005). It was suggested that the mutation alters the properties of the heme, affecting the oxygen sensing system of paraganglionic tissue, leading to chronic hypoxic stimulation and cellular proliferation (Baysal *et al.*, 2000). However, the exact mechanism by which the heme is involved in oxygen sensing is not known.

The membrane anchor domain also interacts with lipid soluble electron carriers known as quinones, which act as either electron acceptors or donors. There are various quinone species with ubiquinone being the molecule that interacts with the majority of SDH enzymes (Table 1.1) (Hägerhäll, 1997). Members of the SQO family have either one or two quinone binding sites. The *E. coli* FRD has two quinone binding sites designated Q_P and Q_D for proximal and distal to the iron-sulfur subunit, respectively. However, the large spatial distance of 25 Å between the two sites makes electron transfer between the sites unlikely; this has led to the suggestion that only Q_P is functional (Iverson *et al.*, 1999). The *E. coli* SDH may have two quinone binding sites, although only one (Q_P) is clearly defined (Yankovskaya *et al.*, 2003). The evidence for two quinone binding sites in eukaryotic enzymes is more convincing (Oyedotun and Lemire, 2001; Sun *et al.*, 2005). Random mutagenesis and inhibitor binding studies in yeast show that there are two distinct sites that are on opposite sides of the membrane anchor domain (Figures 1.4 and 1.5) (Oyedotun and Lemire, 1999; Oyedotun and Lemire, 2001). The two sites have a ten-fold difference in affinity for a dinitrophenol inhibitor and likely correspond to Q_P and Q_D. More recently, two inhibitor binding sites were found in the porcine SDH structure. The ubiquinone analog inhibitor thenoyltrifluoroacetone (TTFA) bound to the Q_P site with a stronger affinity than to the Q_D site (Sun *et al.*, 2005). The TTFA molecule bound at the Q_P site forms more hydrogen bonds and more hydrophobic contacts than the TTFA molecule bound at the Q_D site; the latter molecule interacts mainly through two water molecules (Sun *et al.*, 2005). Interestingly, in the native porcine SDH structure, there is only one ubiquinone molecule bound in the enzyme at the Q_P site. The Q_D site is occupied by a phosphatidylethanolamine molecule (Sun *et al.*, 2005). This is reminiscent of the *E. coli* structure where the analogous location for the Q_D site is occupied by a cardiolipin molecule (Yankovskaya *et al.*, 2003). It could be that the differential binding affinities of the two quinone sites only allows co-crystallization of ubiquinone at the Q_P site. A Q_D site was proposed for the *E. coli* enzyme based on inhibitor binding studies (Yankovskaya *et al.*, 1996).

Since the current evidence favours the presence of two quinone binding sites in the eukaryotic enzyme, questions arise regarding the mechanism of electron transfer to the Q_D site. Direct electron transfer between the two ubiquinone binding sites is not possible due to the large distance of ~26 Å (Sun *et al.*, 2005). The most obvious explanation would be that electron transfer occurs through the heme *b*. Ubiquinone bound at the Q_P site is preferentially reduced compared to the heme because of its higher midpoint potential (Hägerhäll, 1997). However, a fully reduced quinone at the Q_P site could allow reduction of the heme and electron transfer to an ubiquinone molecule bound at the Q_D site (Sun *et al.*, 2005). Many quinone binding proteins have two sites, with one site involved in the stabilization of an ubisemiquinone radical and the second site being in an equilibrium with the quinone pool (Lemire and Oyedotun, 2002). It remains to be seen whether this holds true with SDH. Clearly more work will be required to understand the enigmatic role of the Q_D site.

1.4.5 SDH Homologs

Alternative SDH genes or homologs are present in the *S. cerevisiae* genome and these subunits may be functional. In yeast, homologs for the Sdh1p, Sdh3p and Sdh4p subunits are present; there are no homologs for the Sdh2p subunit. The Sdh1p paralog called Sdh1Bp is 84% identical to Sdh1p and is able to complement the *Δsdh1* disruption mutant when expressed from a multicopy plasmid (Colby *et al.*, 1998). Respiratory growth is significantly slower when the *SDH1B* gene is present in single copy, consistent with an expression level 100-500 fold lower than the *SDH1* gene (Colby *et al.*, 1998). YMR118cp, the Sdh3p homolog is 57% identical to Sdh3p and YLR164wp is 52% identical to Sdh4p (Lemire and Oyedotun, 2002). The *Saccharomyces* Genome Database also identified an additional Sdh4p homolog encoded by the gene *YOR297c* with 36% amino acid sequence identity (Lemire and Oyedotun, 2002). However, *YOR297c* encodes Tim18p, a subunit of the TIM22 inner membrane translocase complex (Kerscher *et al.*, 2000; Koehler *et al.*, 2000). Preliminary evidence suggests that the Sdh3p and Sdh4p homologs can complement the *SDH3* and *SDH4* deletion mutants, respectively when placed on multicopy plasmids (Oyedotun and Lemire, unpublished data). However, deletion of the genes for the homologs does not affect cell viability or respiratory growth (Giaever *et al.*, 2002). This would indicate that these homologs do not play a significant role in normal mitochondrial respiration and may function only under specific growth conditions.

In humans, an Sdh1p homolog may play a role under certain pathological conditions. The human homolog, referred to as SDHA type II, is expressed in various tissues albeit to a much lower level than SDHA (Tomitsuka *et al.*, 2003). Both *SDHA* genes are concurrently expressed in paragangliomas, while SDHA type II is expressed exclusively in a Burkitt's lymphoma cell line (Brière *et al.*, 2005). In addition, a previously identified SDHA Arg-544 to Trp substitution linked to the neurodegenerative disorder Leigh syndrome, has now been associated with the type II subunit, showing the relevance of this subunit to energy transduction in humans (Bourgeron *et al.*, 1995; Tomitsuka *et al.*, 2003).

1.4.6 Assembly of SDH

The assembly of MRC enzymes is an intricate process because these complexes are integral membrane proteins and contain a significant number of subunits and cofactors. In eukaryotes, assembly is further complicated by the fact that the majority of subunits are nuclear encoded and translated in the cytosol. These proteins are imported into mitochondria in an unfolded state and folded once they reach their destination (Neupert, 1997). Four of the five members of the MRC also contain subunits encoded by the mtDNA and these proteins are synthesized in the mitochondrial matrix (Koehler, 2004). In order for proper assembly of these enzymes to occur, there must be a co-ordination between both genomes to ensure comparable rates of synthesis for enzyme subunits. These subunits are then processed, properly folded and assembled into holoenzymes (Straffon *et al.*, 1998; Vogel *et al.*, 2005). In addition, cofactors and prosthetic groups such as iron-sulfur clusters and heme must be synthesized and incorporated in a controlled fashion (Muhlenhoff and Lill, 2000). Because of their complexity, the proper formation and function of each of the individual MRC holoenzymes requires a number of chaperones and assembly factors. Assembly factors or chaperones are proteins that interact transiently with components of the MRC enzymes but do not form a part of the final complex. A large group of accessory proteins has been identified that aid in the proper assembly of these enzymes (Kermorgant *et al.*, 1997; Suzuki *et al.*, 1997; Cruciat *et al.*, 1999; Nijtmans *et al.*, 2000; Khalimonchuk and Rödel, 2005; Fontanesi *et al.*, 2006). Chaperones may be specific to a particular complex or act as general chaperones mediating the assembly of multiple complexes. Although a number of proteins have been identified as assembly factors, particularly for complex IV, it is believed many remain to be identified.

It is increasingly obvious that the assembly of the eukaryotic SDH is not a spontaneous event but rather a complex process involving many coordinated steps requiring chaperones or assembly factors. Elucidating the mechanism and various steps in the assembly of MRC enzymes has been and continues to be a major focus in the understanding of mitochondrial biogenesis. The *S. cerevisiae* SDH, consisting of only four subunits, is a good model for studying the assembly of respiratory chain enzymes because of its simplicity in comparison to other MRC enzymes. So far, two assembly factors, Abc1p and Tcm62p, which will be discussed in the next two chapters, have been implicated in the assembly of SDH in yeast.

The *ABC1* gene was originally isolated as a multicopy suppressor of a translation defect in the cytochrome *b* mRNA (Bousquet *et al.*, 1991). Abc1p is a mitochondrial protein with a high degree of evolutionary conservation across species. It has weak sequence similarity (16.7%) to the yeast chaperonin Hsp60. Abc1p may also be a chaperone for complex III biogenesis, although there is no direct evidence of an interaction between Abc1p and cytochrome *b* (Bousquet *et al.*, 1991; Brasseur *et al.*, 1997). Deletion of the gene leads to deficiencies in complex II, III and IV, suggesting that its role is not limited to a particular complex. *ABC1* mutants demonstrate a specific loss of almost 90% of the spectrally detectable heme in complex II with only minor losses of total cytochrome *b* in complex III and no differences in the levels of cytochrome aa_3. In addition, all three complexes become thermolabile in the absence of Abc1p. Complexes

II and IV may undergo Abc1p-dependent structural alterations (Brasseur *et al.*, 1997), or alternatively, the changes in the properties of these complexes may be an indirect result of effects on the structure of complex III. Complexes II, III and IV are thought to be in a supercomplex in the yeast mitochondrial respiratory chain (Boumans *et al.*, 1998). Alterations to the structure of complex III have also been shown to cause adverse effects on SDH activity (Boumans *et al.*, 1997). However, SDH is not detected in supercomplexes isolated from yeast mitochondria using blue native gel electrophoresis (Schägger and Pfeiffer, 2000). Abclp may also have an indirect role in ubiquinone biosynthesis (Do *et al.*, 2001). Abc1p contains motifs found in eukaryotic protein kinases; it may regulate a step of ubiquinone biosynthesis by phosphorylating a biosynthetic enzyme (Poon *et al.*, 2000). This would suggest that ubiquinone plays a role in the biogenesis of SDH. This notion is supported by the observation that the loss of the *COQ5* gene, which encodes a methyltransferase in the yeast ubiquinone biosynthetic pathway, also causes defects in SDH assembly (Dibrov *et al.*, 1997).

The temporal order of assembly of SDH is perhaps the least complicated of all MRC enzymes because it is the simplest in terms of subunit composition. In addition, there are no mtDNA subunits, which would add an additional layer of complexity to the process. Generally, eukaryotic SDH subunits are translated as precursor proteins containing cleavable N-terminal mitochondrial presequences (Ackrell *et al.*, 1992; Lemire and Oyedotun, 2002). In contrast, the bacterial versions of these subunits do not contain these presequences (Hägerhäll, 1997). Once imported into the mitochondrion, the targeting sequences are cleaved by proteases, including the mitochondrial processing peptidase and the mitochondrial intermediate peptidase (Branda and Isaya, 1995). All four subunits of the yeast enzyme have their N-termini localized to the mitochondrial matrix and are processed by one or both of these proteases (Lemire and Oyedotun, 2002). Once processed, the eukaryotic subunits are thought to exhibit similar properties to their bacterial counterparts. Much of the evidence regarding SDH assembly has been obtained from bacterial systems and it is assumed that the assembly of metazoan enzymes follows a similar pathway (Hederstedt and Ohnishi, 1992; Hägerhäll, 1997; Scheffler, 1998). The available data indicate that SDH assembly starts with the incorporation of prosthetic groups into the appropriate subunits. This is followed by subunit association, with the anchor subunits first being incorporated into the membrane, leading to the formation of a functional enzyme.

Incorporation of prosthetic groups should be an early step in the assembly process because incorporation into fully folded subunits would be a difficult process. For the FAD and iron-sulfur clusters, evidence suggests that their incorporation occurs prior to the assembly of the catalytic dimer or membrane docking (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992) . Flavinylation is thought to be autocatalytic; accessory proteins that would mediate this reaction are not anticipated (Mewies *et al.*, 1998). In yeast, the chaperonin Hsp60 interacts with Sdh1p but is not required for flavinylation to occur (Robinson and Lemire, 1996). The attachment of the FAD group to Sdh1p occurs after processing indicating that the presequence prevents the flavinylation reaction from occurring, possibly by hindering the folding of the preprotein until it reaches the matrix (Robinson and Lemire, 1996; Robinson and Lemire, 1996). This suggests that flavinylation requires the presence of the entire polypeptide in a properly folded conformation. Evidence also indicates that flavinylation occurs prior to the interaction of flavoprotein subunit with other subunits. When assembly of the *B. subtilis* SDH is blocked by either heme depletion or by the absence of a functional anchor subunit, SdhA containing covalent FAD accumulates in the cytosol (Hederstedt and Rutberg, 1980; Hederstedt *et al.*, 1982). Similar experiments using *E. coli* and *W. succinogenes* FRD show that FrdA containing covalent FAD can be independently expressed (Unden and Kröger, 1981; Cole *et al.*, 1985; Cecchini *et al.*, 1986).

The iron-sulfur clusters are first synthesized in the mitochondrion prior to their incorporation into Sdh2p. The synthesis of iron-sulfur clusters is an essential process, which requires at least 10 different proteins (Lill and Kispal, 2000). Mutations in many of these proteins cause defects in SDH assembly, most likely resulting from structural instability of the Sdh2p subunit when it lacks incorporated Fe-S clusters and is unable to assemble into the catalytic dimer (Lemire and Oyedotun, 2002). The depletion of Yfh1p (yeast frataxin homolog), which is involved in iron homeostasis, results in the accumulation of iron in mitochondria and an associated loss of SDH activity (Babcock *et al.*, 1997; Rötig *et al.*, 1997). Yfh1p physically interacts with Sdh1p and Sdh2p, suggesting that it may play a role in SDH assembly (González-Cabo *et al.*, 2005). Yfh1p also interacts with components of the iron-sulfur biogenesis pathway (Muhlenhoff *et al.*, 2002). SDH activity is also lost and the SDH subunits are degraded in the absence of Nfs1p, a cysteine desulfurase (Kispal *et al.*, 1999; Li *et al.*, 1999). Deletions of the *ISU1* and *ISU2* genes are lethal and cause decreased expression of the Sdh1p and Sdh2p subunits and a marked reduction of SDH activity (Schilke *et al.*, 1999). The Isu1p and Isu2p proteins play a crucial role in biogenesis by serving as a scaffold for *de novo* assembly of Fe-S clusters (Muhlenhoff *et al.*, 2003). Mutations to other proteins such as

Ssq1p (an Hsp70-type chaperone required for assembly of Fe-S clusters into proteins after cluster synthesis) and Jac1p (a co-chaperone of Ssq1p) also lead to the loss of SDH function (Strain *et al.*, 1998; Vickery and Cupp-Vickery, 2007). These proteins are thought to be involved in the transfer and insertion of Fe-S clusters into Sdh2p (Muhlenhoff *et al.*, 2003). All of these proteins function in the general Fe-S cluster biogenesis pathway and whether there are additional SDH specific assembly factors remains to be determined. Much like the covalent attachment of FAD, the incorporation of Fe-S clusters into Sdh2p is believed to precede any subunit interactions.

The role of heme in the assembly of SDH remains controversial. Using *B. subtilis* heme-deficient mutants, it was shown that heme insertion is an essential step in SDH assembly. The absence of heme leads to the accumulation of the catalytic dimer in the cytoplasm. When heme becomes available, assembly from preformed subunits proceeds (Hederstedt and Rutberg, 1980) Mutants in the membrane anchor domain lacking one or both hemes cannot assemble properly, resulting in the accumulation of inactive SdhA and SdhB subunits in the cytosol (Fridén and Hederstedt, 1990). Similarly, the cytoplasmic accumulation of the SDH catalytic domain was also observed in *E. coli* heme synthesis mutants (Nakamura *et al.*, 1996; Nihéi *et al.*, 2001). These results support the importance of heme in the assembly of enzyme. In contrast, the bovine SDH lacking heme can be reconstituted *in vitro* (Hatefi and Galante, 1980; Yu and Yu, 1980; Ackrell *et al.*, 1992). The yeast SDH can also assemble efficiently in the absence of heme (Oyedotun *et al.*, 2007). One explanation for the discrepancies may be that heme *b* is only required for the initial steps in the assembly of SDH into the membrane. Heme may be required for the proper formation of the anchor domain and once that occurs, its presence is not obligatory. Alternatively, the assembly pathways of the bacterial and the mitochondrial enzymes may differ in their requirements for heme.

Insertion of the anchor subunits into the membrane is believed to constitute an early step in the subunit assembly of the enzyme. Pulse-chase experiments with the *B. subtilis* SDH show that the SdhC anchor subunit is rapidly inserted into the membrane after synthesis (Hederstedt and Rutberg, 1980). Mutants lacking either subunit of the catalytic dimer continued to correctly insert the anchor subunit (Hederstedt *et al.*, 1982). In addition, evidence from bacterial systems shows that in the absence of the membrane anchor subunits, there is an accumulation of the catalytic domain within the cytosol (Hederstedt and Rutberg, 1980; Fridén and Hederstedt, 1990; Hederstedt and Ohnishi, 1992). Membrane insertion of the anchor proteins is an initial step in SDH subunit assembly, consistent with the established docking role of the membrane anchor domain (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992; Scheffler, 1998).

The mechanism of attachment of the catalytic domain to the anchor domain remains unresolved. There are two views regarding this issue, with the first consisting of a concerted docking of subunits to the membrane anchor. The other proposed mechanism suggests that the subunits comprising the catalytic domain dimerize prior to membrane docking. Evidence has been presented for both mechanisms. Pulse-chase experiments in *E. coli* suggest that FrdA attaches to the membrane before the docking of FrdB (Latour and Weiner, 1989). Also, in absence of the anchor subunit, the catalytic domain subunits of the *B. subtilis* SDH are found as monomers in the cytosol (Hederstedt *et al.*, 1982). In contrast, there are several observations that are inconsistent with separate docking of the catalytic subunits. In *E. coli*, the FrdA and FrdB subunits are able to form a functional heterodimer when expressed in the absence of the membrane anchor subunits (Hederstedt and Rutberg, 1980; Lemire *et al.*, 1982). Also, the *E. coli* FRD and the mammalian SDH can be reconstituted into functional enzymes by adding preassembled catalytic domains to their respective membrane domains (Cecchini *et al.*, 1986; Ackrell *et al.*, 1992). In yeast, the absence of either Sdh1p or Sdh2p leads to the rapid degradation of the other subunit, suggesting the existence of a preassembled catalytic domain is required for stability (Schmidt *et al.*, 1992). Considering the evidence, the docking of preassembled catalytic domain seems most likely.

1.5 The Mitochondrial Respiratory Chain and Mitochondrial Diseases.

It is becoming increasingly clear that defective mitochondria contribute to many diseases and degenerative conditions. Over 100 heritable disorders have been reported and attributed to defects in a number of the known nuclear-encoded mitochondrial proteins in humans (Steinmetz *et al.*, 2002). The MRC is the primary source of ATP generation in cells and it is not surprising that mutations that impair its function produce deleterious consequences (Wallace, 1999; Munnich and Rustin, 2001). As expected, many mutations in genes encoding structural components of the MRC resulting in a clinical presentation have been characterized (Johns and Neufeld, 1993; Majander *et al.*, 1997; Baysal *et al.*, 2001; Triepels *et al.*, 2001). However, there are numerous cases for which symptoms of mitochondrial diseases are present but mutations in MRC structural genes cannot be found. In some of these cases, defects in other proteins involved in the biogenesis, assembly or maintenance of the MRC have been detected (Parfait *et al.*, 1997; Parfait *et al.*, 2000; De Meirleir *et al.*, 2003; De Meirleir *et al.*, 2004). This is highlighted by the fact that a number of assembly proteins are associated with complex IV deficiencies. Mutations in genes such as *SURF1, SCO1, SCO2* and *COX 10* result in mitochondrial disease with reduced complex IV activity (Zhu *et al.*, 1998; Papadopoulou *et al.*, 1999; Valnot *et al.*, 2000; Valnot *et al.*, 2000; Barrientos *et al.*, 2002). Shy1p, the yeast homolog of Surf1, associates with assembly intermediate subcomplexes of complex IV (Mick *et al.*, 2007). Sco1p and Sco2p function to deliver copper to this enzyme (Leary *et al.*, 2004; Horng *et al.*, 2005) while Cox10p catalyzes the first step in the conversion of protoheme to the heme A prosthetic group required for cytochrome *c* oxidase activity (Tzagoloff *et al.*, 1993).

In humans, mutations in the SDH genes can result in a variety of clinical phenotypes including optic atrophy, ataxia, muscle weakness, myopathy, tumour formation and Leigh syndrome (a degenerative disorder of the central nervous system) (Sugimoto *et al.*, 2000; Baysal *et al.*, 2001; Rustin *et al.*, 2002; Rustin and Rötig, 2002). Leigh syndrome is a common mitochondrial respiratory chain disorder and is associated with defects in complexes I, III and IV as well (DiMauro and Schon, 2003). However, there is one reported case of a mitochondrial disorder characterized by SDH deficiency with an absence of mutations in the *SDHA-D* genes (Parfait *et al.*, 2000). This observation suggests that mutations in proteins involved in the synthesis, assembly or maintenance of SDH may be responsible.

1.6 Mitochondrial Biology

1.6.1 Apoptosis

Mitochondria are central to the development of disease phenotypes mainly because of two cellular processes: apoptosis and the generation of reactive oxygen species (ROS) (the latter will be discussed in subsection 1.6.2). Apoptosis is a controlled and programmed mechanism of cellular death used for tissue development, removal of damaged cells and cellular homeostasis in multicellular organisms (Joza *et al.*, 2001). Defects in this essential process can result in oncogenesis and the continued proliferation of damaged or mutated cells.

Hallmarks of apoptosis include chromatin condensation, DNA fragmentation, membrane blebbing, cytosolic acidification, extracellular exposure of phosphatidylserine and the permeabilization of the mitochondrial outer membrane (Kerr *et al.*, 1972; Martin *et al.*, 1995; Clifford *et al.*, 1996; Hengartner, 2000). This latter feature is a major event in the apoptotic process and causes the release of proteins from the intermembrane space into the cytosol (Waterhouse *et al.*, 2002). Several of these proteins, including cytochrome *c*, apoptosis-inducing factor (AIF), Smac/DIABLO, Omi/Htra2 and EndoG have very specific roles in the apoptotic process (Susin *et al.*, 1999; Du *et al.*, 2000; Verhagen *et al.*, 2000; Li *et al.*, 2001; Suzuki *et al.*, 2001).

Permeabilization of the mitochondrial outer membrane can occur by two distinct mechanisms. The first involves the recruitment of members of the Bcl-2 family of proteins to the outer mitochondrial membrane in response to an apoptotic signal. Pro-apoptotic members of this family such as Bax and Bid integrate into the outer membrane (Epand *et al.*, 2002; Annis *et al.*, 2005). The protein Bak does not require integration as it exists as an integral membrane protein of the outer membrane (Cheng *et al.*, 2003). After integration, conformational changes in the proteins facilitate their oligomerization and lead to pore formation (Basanez *et al.*, 1999; Annis *et al.*, 2005), allowing the proteinaceous contents of the intermembrane space to escape into the cytosol. Anti-apoptotic members such as Bcl-2 and Bcl-x_L function to inhibit the progression of apoptosis. These proteins can bind to pro-apoptotic members such as Bax and Bad, sequestering them and thus, inhibiting their function (Kelekar *et al.*, 1997; Dlugosz *et al.*, 2006).

During apoptosis, a morphological change occurs with the fragmentation of the reticular mitochondrial network, suggesting that the apoptosis and the mitochondrial fusion/fission machinery interact. Bax co-localizes with Drp1 and Mfn2, proteins involved in mitochondrial fission and fusion respectively (Karbowski *et al.*, 2002). Bak was also shown to interact with mitofusins (Brooks *et al.*, 2007). Evidence suggests that pro-apoptotic proteins play a role in inducing mitochondrial fragmentation and mitochondrial injury during apoptosis. In agreement with this finding, mitochondrial fission proteins, such as Dnm1p and Mdv1p regulate programmed cell death in yeast (Fannjiang *et al.*, 2004). Expression of the antiapoptotic Bcl-2 protein in mammalian cells promotes mitochondrial fusion, and CED-9, the *C. elegans* homolog of Bcl-2 immunoprecipitates with Mfn2 (Delivani *et al.*, 2006). Bcl-x_L also promotes mitochondrial fusion, consistent with an observation that Bcl-x_L interacts with Mfn2 (Delivani *et al.*, 2006). Conversely, other studies have investigated the roles of fission and fusion proteins in apoptosis. These include OPA1 (Mgm1p in yeast), Fis1 and PARL (Rbd1p in yeast; a rhomboid protease of the inner membrane), which are thought to facilitate the efficient release of intermembrane space proteins by remodeling inner membrane cristae (Fannjiang *et al.*, 2004; Youle and Karbowski, 2005; Cipolat *et al.*, 2006; Frezza *et al.*, 2006).

The second mechanism of outer membrane permeabilization involves the formation of a large protein complex known as the mitochondrial permeability transition pore (MPT). The structure and composition of the MPT are controversial. The favored model of MPT is a complex of the inner membrane protein adenine nucleotide translocase (ANT), the outer membrane protein voltage-dependent anion channel (VDAC or porin), along with cyclophilin D from the matrix and other proteins, such as the benzodiazepine receptor, hexokinase and creatine kinase (Beutner *et al.*, 1998; Budd *et al.*, 2000; Kim *et al.*, 2003; Newmeyer and Ferguson-Miller, 2003). Formation and sustained opening of the MPT leads to the equilibration of solute concentrations between the matrix and the cytosol. In addition, it dissipates the membrane potential and inhibits mitochondrial respiratory chain function and protein transport. The equilibration of ion concentrations results in the swelling of the matrix space and rupture and permeabilization of the outer membrane (Skulachev, 1996; Scorrano *et al.*, 2002). Outer membrane rupture releases intermembrane space proteins such as cytochrome *c* that facilitate downstream apoptotic events.

Once in the cytosol, cytochrome *c* binds to apoptotic peptidase activating factor 1 (Apaf-1), causing its oligomerization and the subsequent formation of the apoptosome (Wang, 2001; Acehan *et al.*, 2002). The apoptosome recruits the apoptotic proteinase procaspase-9 and facilitates the activation of the zymogen. After cleavage, mature caspase-9 remains bound to the apoptosome, where it is able to activate executioner caspases, such as caspases 3 and 7 (Rodriguez and Lazebnik, 1999). The activated executioner caspases cleave a number of intracellular targets, including enzymes of the MRC, eventually leading to cell death. Caspase-3 disrupts the functions of complexes I and II, presumably by proteolytic cleavage of subunits of the complexes, resulting in the loss of membrane potential and ROS generation (Ricci *et al.*, 2003). These findings revealed an interesting mechanism where following cytochrome *c* release, the activation of caspases feeds back on the permeabilized mitochondria to damage mitochondrial function (loss of membrane potential) and generate ROS through the effects of caspases on complexes I and II (Ricci *et al.*, 2003; Ricci *et al.*, 2004). Apoptosis and the generation of ROS by the members of the MRC are closely connected, since ROS participate in both early and late steps of apoptosis (Madeo *et al.*, 1999). At high levels, ROS are deleterious to cells, leading to programmed cell death (Raha and Robinson, 2001).

1.6.2 Reactive Oxygen Species

At low levels, ROS serve many important cellular functions. These include acting as a second messenger, as an anti-bacterial agent and as a growth stimulant (Ramsey and Sharpless, 2006). Mitochondria are the major sites of ROS production within the cell due to their involvement in electron transport and are often one of the first cellular components to suffer from ROS mediated damage (Harman, 1956; Hillered and Ernster, 1983). It is estimated that under physiological conditions, 1 - 2% of the oxygen consumed by the MRC is not fully reduced to water but rather is partially reduced to superoxide anion ($O_2^{\cdot -}$) (Fridovich, 1995; Raha and Robinson, 2000). Although relatively unreactive itself, superoxide can give rise to other more damaging ROS. Superoxide can directly damage iron-sulfur clusters in enzymes, such as aconitase (Raha and Robinson, 2000). It can be scavenged by cytochrome *c* or alternatively converted into the more stable, membrane permeable molecule hydrogen peroxide (H_2O_2) (Fridovich, 1995). This reaction is catalyzed by the copper/zinc superoxide dismutase (Cu/Zn SOD) and the manganese superoxide dismutase (MnSOD), which reside in the cytoplasm and mitochondrial matrix, respectively (Fridovich, 1995). Hydrogen peroxide is then converted into water by the glutathione peroxidase-mediated oxidation of glutathione (Esworthy *et al.*, 1997). However, if it escapes the cell's detoxification machinery, hydrogen peroxide can generate the highly reactive hydroxyl radical ($OH^{\cdot}$) in the presence of ferrous iron (Fe^{2+}) via Fenton chemistry (Pryor, 1986; Raha and Robinson, 2000). The hydroxyl radical is highly toxic, causing lipid peroxidation, protein oxidation, DNA damage, reduced MRC function and decreased ATP synthesis (Beckman and Koppenol, 1996). Under pathological conditions or conditions of cell stress, such as MRC dysfunction, ROS production can overwhelm cellular detoxification mechanisms resulting in cellular injury and eventually cell death (Raha and Robinson, 2001).

Traditionally, complexes I and III are thought to be sites of ROS generation, especially under conditions of high membrane potential (Korshunov *et al.*, 1997; Raha and Robinson, 2000; Staniek *et al.*, 2002). However, evidence from *S. cerevisiae* and *C. elegans* suggests that complex II can also generate ROS (Ishii *et al.*, 1998; Senoo-Matsuda *et al.*, 2001; Guo and Lemire, 2003). Complex II must couple the one-electron redox chemistry of iron-sulfur centers to two-electron acceptors/donors such as flavins and quinones. Electron transfer must proceed in two separate steps resulting in the formation of an ubisemiquinone intermediate (Lemire and Oyedotun, 2002). It is believed that the ubisemiquinone radical is the principle electron donor for the generation of superoxide (Raha and Robinson, 2000). Complex II mutations that enhance the stability of the ubisemiquinone radical could result in increased free radical production (Ishii *et al.*, 1998). Mutations that expose either the iron-sulfur clusters or the quinone binding sites to molecular oxygen could also directly enhance the formation of superoxide (Cecchini, 2003).

1.7 Thesis Objective

Over the past few decades, significant advances in our understanding of SDH structure and function have been made. The majority of the information that has been acquired comes from the study of mammalian and microbial model organisms. Both have their advantages and limitations. Studies with the mammalian enzyme are pertinent to human biology but lack the capacity for genetic manipulation. Microbial model systems are amenable to genetic approaches that enable the study of structure-function relationships. However, differences in the microbial SDHs make it difficult to translate some of the information gathered in these systems to the human enzyme.

S. cerevisiae offers the advantages of studying a mitochondrial enzyme in the context of a well developed genetic model system. It is a simple unicellular eukaryote with short generation times and a fully sequenced genome. It is highly amenable to biochemical, genetic and cytological approaches. The generation of disrupted alleles and the introduction of modified genes can be performed quickly and with relative ease. Many features important to higher eukaryotes are conserved in yeast, as indicated by the large number of genes with human disease homologs (Foury, 1997; Steinmetz *et al.*, 2002). Another important aspect in the context of mitochondrial research is that *S. cerevisiae* is a facultative anaerobe. Yeast are able to grow without oxidative phosphorylation; the cell's energy requirements are fulfilled by substrate level phosphorylation (Kovác *et al.*, 1967). This facilitates the study of mitochondrial proteins involved in oxidative phosphorylation. Any insights gained from yeast studies are likely to be highly relevant to SDHs in all eukaryotes because of the high degree of conservation of SDH enzymes and of MRC components in general.

Despite the significant progress that has been made, many questions regarding SDH function and biogenesis remain. In particular, many questions involving the assembly of the enzyme remain unanswered. So far, one SDH-specific chaperone called Tcm62p has been identified. My goal was to study the structure and function of Tcm62p in order to gain insight into its role in SDH assembly. In chapter 2, I examined the structure of the Tcm62p complex. I show that Tcm62p is a component of two multiprotein complexes that reside in the mitochondrial inner membrane. In chapter 3, a potential interaction of the Tcm62p complex with another chaperone, the prohibitin complex, was examined. Although previous evidence suggested an interaction, I did not detect any interaction between the two chaperones.

Another area of increasing importance is the involvement of SDH in human pathologies. In chapter 4, I modeled human cancer mutations in the yeast SDH to better understand the molecular mechanisms of these disorders. I provide evidence that ROS production and succinate accumulation are both occurring during SDH dysfunction and that both processes may contribute to tumorigenesis in humans.

In the final chapter of the thesis, I will discuss potential avenues of research that may provide insight into the role of chaperones such as Tcm62p in the biogenesis of SDH. I will also discuss future directions that examine aspects of SDH structure and function not yet explored. These new areas of research will expand our knowledge and understanding into the biogenesis of SDH and the role of this enzyme in mitochondrial biology and involvement in human mitochondrial disorders.

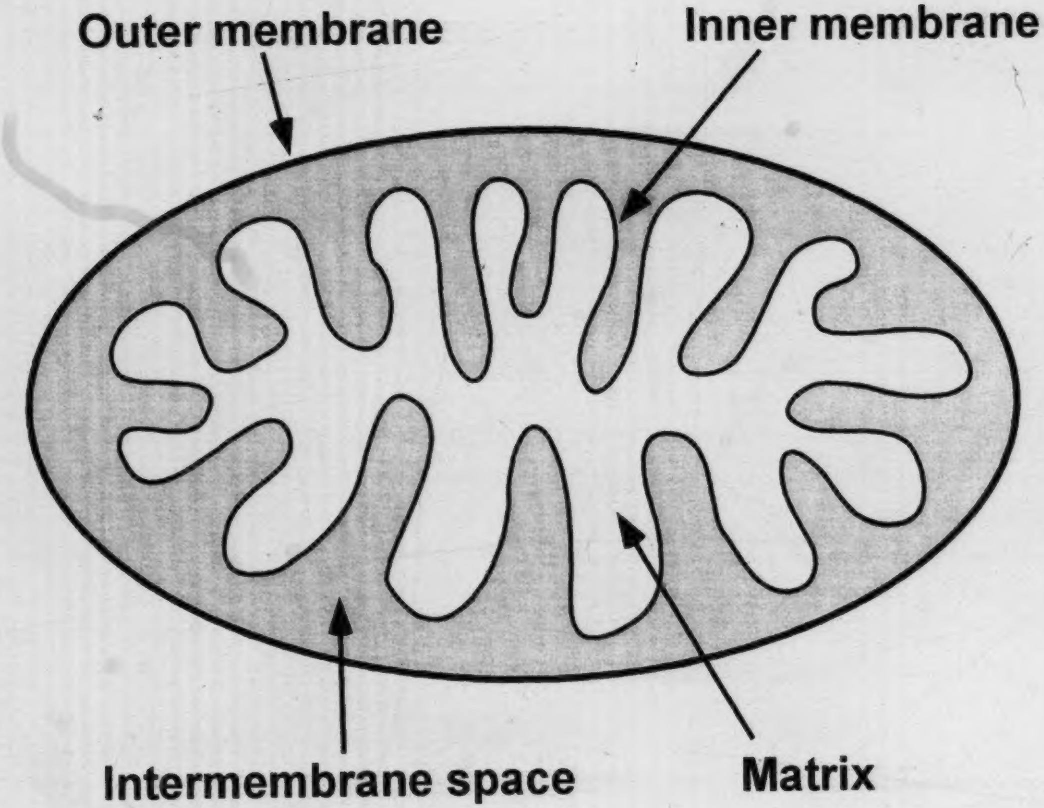
Outer membrane
Inner membrane
Intermembrane space
Matrix

Figure 1.1 Schematic diagram of mitochondrial ultrastructure.

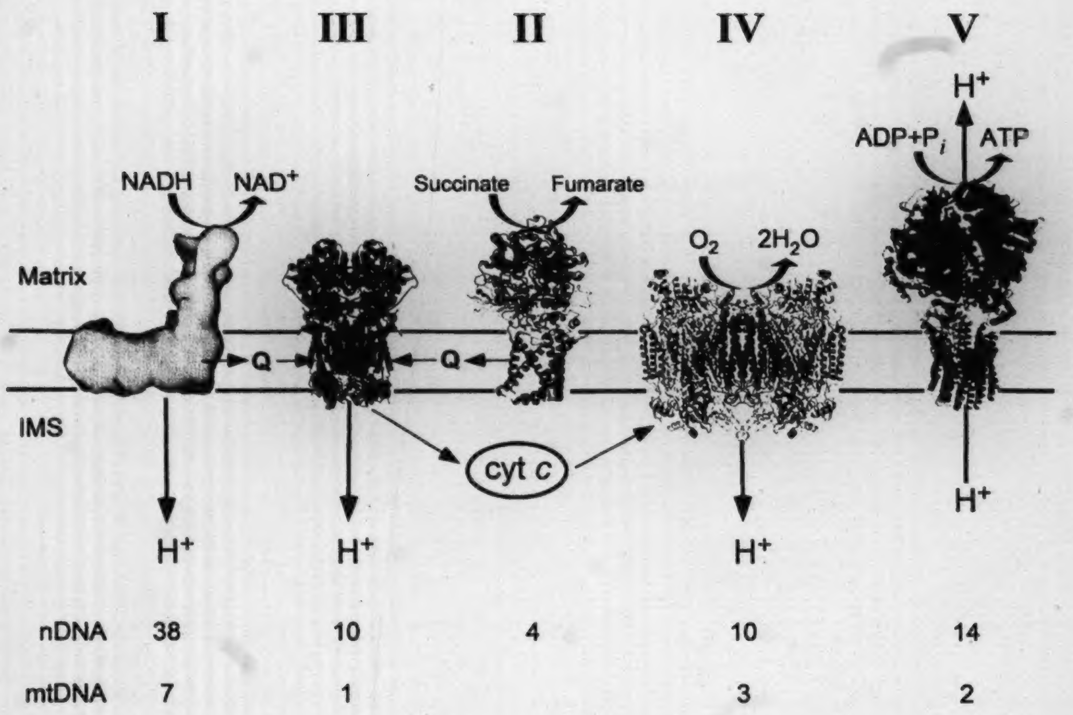
I
III
II
IV
V
H+
ADP+Pi
ATP
NADH
NAD+
Succinate
Fumarate
O2
2H2O
Matrix
Q
Q
IMS
cyt c
H+
H+
H+
H+
nDNA 38 10 4 10 14
mtDNA 7 1 3 2

Figure 1.2 The mammalian mitochondrial respiratory chain (MRC). The three-dimensional structures of the MRC complexes (I-V) are shown in the mitochondrial inner membrane. Shown below are the nuclear and mitochondrial genome contributions to the subunit compositions of each complex. Arrows represent the flow of electrons. The abbreviations used are: Q, ubiquinone; cyt *c*, cytochrome *c*; IMS, intermembrane space.

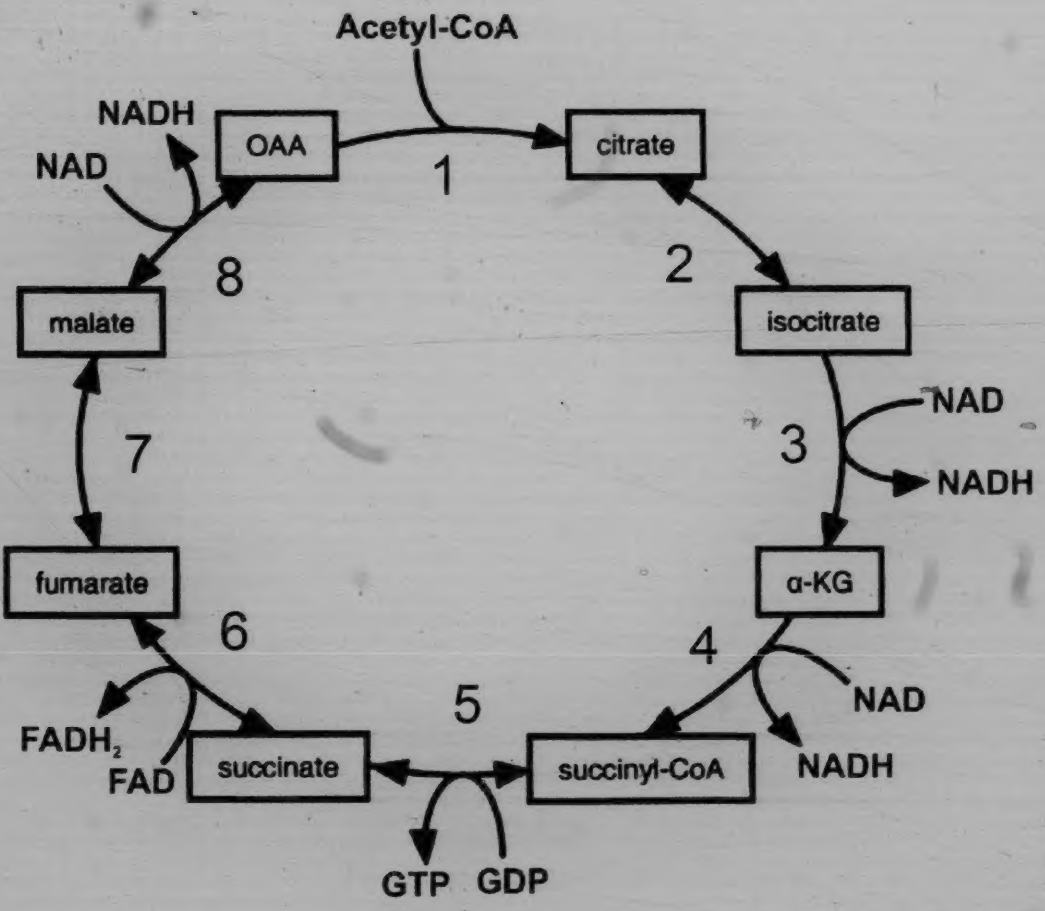
Acetyl-CoA
NADH
OAA
citrate
NAD
1
8
2
malate
isocitrate
NAD
3
7
NADH
fumarate
α-KG
6
4
5
NAD
FADH2
FAD
succinate
succinyl-CoA
NADH
GTP
GDP

Figure 1.3 The Krebs cycle. The enzymes of the Krebs cycle are: 1, citrate synthase; 2, aconitase; 3, isocitrate dehydrogenase; 4, α-ketoglutarate dehydrogenase; 5, succinyl-CoA synthetase; 6, succinate dehydrogenase; 7, fumarase; 8 malate dehydrogenase. The abbreviations used are: OAA, oxaloacetate; α-KG, α-ketoglutarate. Adapted from Przybyla-Zawislak *et al.* (1999)

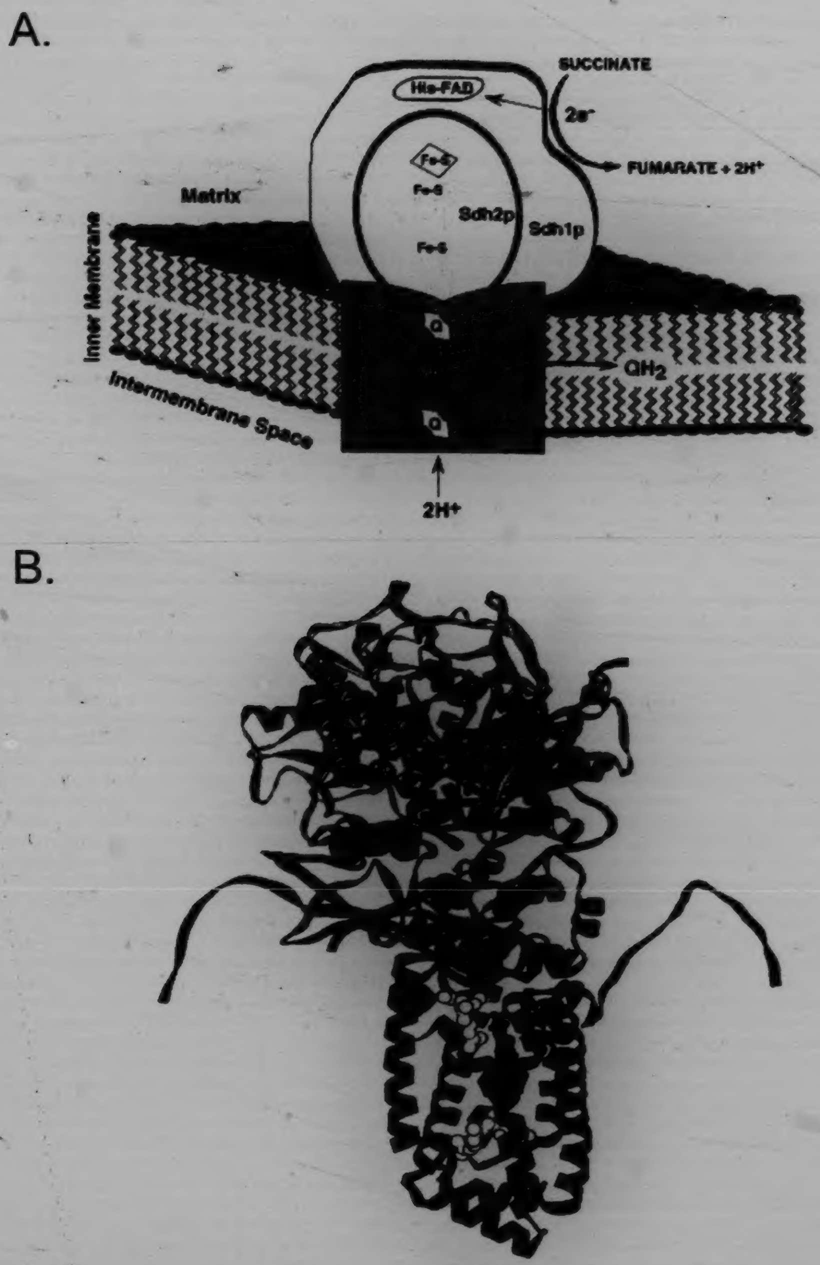
A.
SUCCINATE
His-FAD
2e⁻
FUMARATE + 2H⁺
Fe-S
Fe-S
Fe-S
Matrix
Sdh2p
Sdh1p
Inner Membrane
Q
QH₂
Q
Intermembrane Space
2H⁺
B.

Figure 1.4 Structure of the *S. cerevisiae* complex II. *A*, Schematic representation of SDH showing the relative locations of the four subunits, cofactors and electron flow. Adapted from Lemire and Oyedotun (2002). *B*, Modeled structure of the yeast SDH shown as a ribbon representation. Sdh1p is shown in gold, Sdh2p in green, Sdh3p in red and Sdh4p in blue. FAD is shown in light purple, Fe-S clusters are shown in light blue and red, ubiquinones are shown in yellow and heme *b* is shown in dark purple. Adapted from Oyedotun and Lemire (2004).

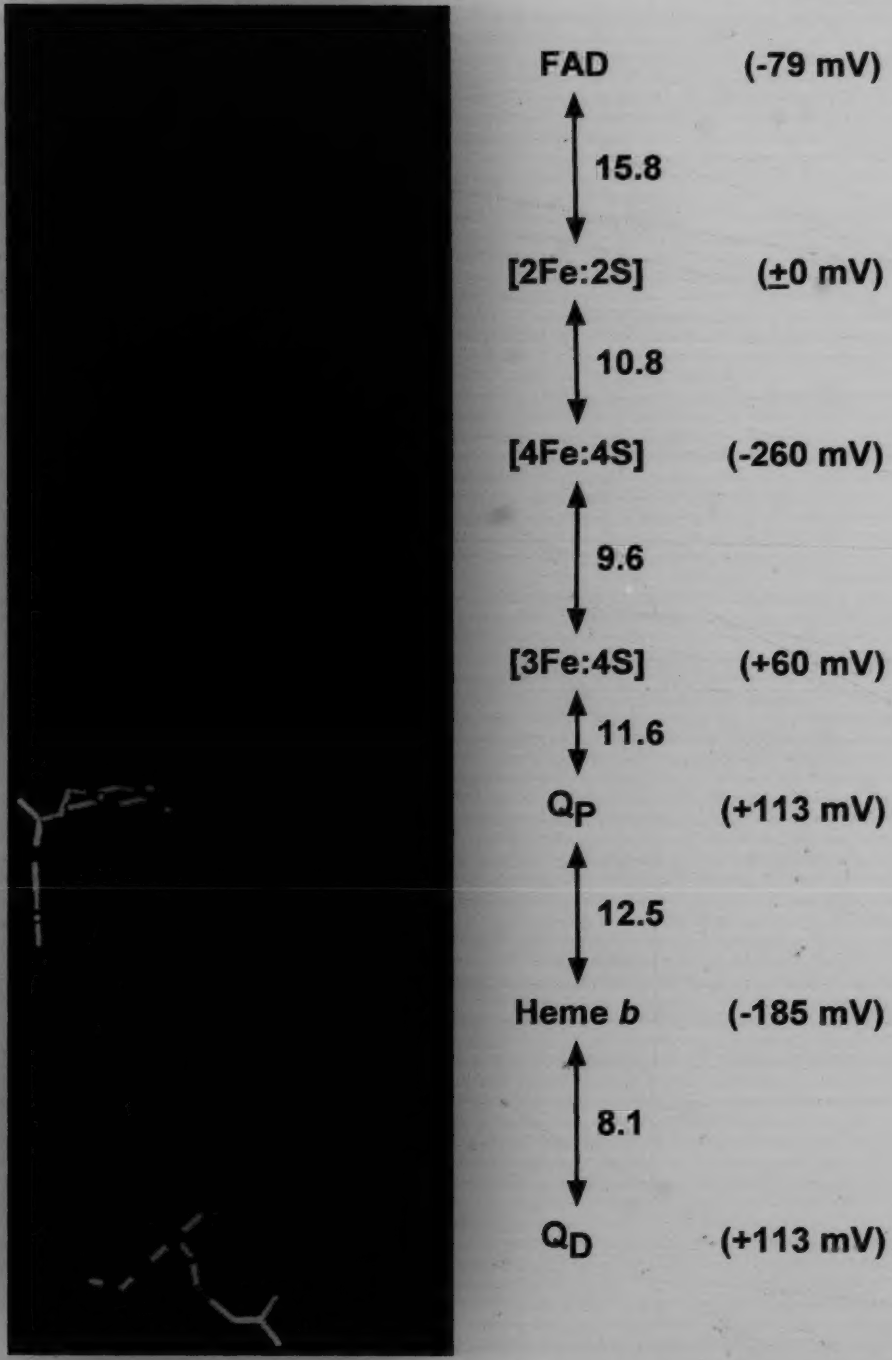
FAD (-79 mV)
15.8
[2Fe:2S] (±0 mV)
10.8
[4Fe:4S] (-260 mV)
9.6
[3Fe:4S] (+60 mV)
11.6
Q_P (+113 mV)
12.5
Heme *b* (-185 mV)
8.1
Q_D (+113 mV)

Figure 1.5 Cofactor locations and electron transfer pathway within the *S. cerevisiae* complex II. Edge-to-edge distances in angstroms between the cofactors determined from the yeast model are indicated. Midpoint redox potentials determined for the bovine SDH are also indicated. Adapted from Oyedotun and Lemire (2004) and Hägerhäll (1997).

TABLE 1.1

Properties of hemes and quinones from selected FRDs and SDHs.

Enzyme	Classification	TMH	Heme *b* (mV)	Quinone
T. acidophilum SDH	Type A	6	b_H: (+75) b_L: (-150)	MMQ (-74)
B. subtilis SDH	Type B	5	b_H: (+65) b_L: (-95)	MQ (-74)
W. succinogenes FRD	Type B	5	b_H: (+20) b_L: (-200)	MQ (-74)
A. suum FRD	Type C	6	(-34)	RQ (-63)
B. taurus SDH	Type C	6	(-185)	UQ (+113)
E. coli SDH	Type C	6	(+36)	UQ (+90)
P. denitrificans SDH	Type C	6	(-176)	UQ (+113)
S. cerevisiae SDH	Type C	6	Present	UQ (+113)
E. coli FRD	Type D	6	Not Present	MQ (-74)

The data are adapted from Ackrell (1992), Hägerhäll (1997) and Hederstedt (2002). The abbreviations used are: SDH, succinate dehydrogenase; FRD, fumarate reductase; TMH, transmembrane helices; b_H, high potential heme; b_L, low potential heme; MMQ, methylated menaquinone (*Thermoplasma* quinone); MQ, menaquinone; RQ, rhodoquinone; UQ, ubiquinone. The midpoint potential for hemes and quinones are indicated in parentheses.

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CHAPTER 2

Structure and Function of Tcm62p, a Chaperone Required for the Assembly of the *Saccharomyces cerevisiae* Succinate Dehydrogenase

2.1 Introduction

Biogenesis of the MRC is an extremely intricate process requiring the participation of chaperones or assembly factors. Currently, Tcm62p is the only protein identified as being specifically involved in SDH assembly. There are several lines of evidence to support its role as an SDH-specific chaperone. The *TCM62* gene, designated for tricarboxylic acid cycle mutant, was originally isolated in a genetic screen for mutants with a loss of SDH activity (Dibrov *et al.*, 1998). Disruption of the *TCM62* gene causes respiration deficiency and an almost complete loss of SDH-dependent enzymatic activity with only minor effects on the activities of the NADH or the glycerol phosphate dehydrogenases. In the absence of Tcm62p, SDH is not assembled and membrane-associated Sdh1p and Sdh2p are not observed. Blue native gel electrophoresis (BN-PAGE) revealed the comigration of Tcm62p and the SDH holoenzyme in a 200-kDa complex. When overexpressed, Tcm62p forms intramitochondrial inclusion bodies that contain Sdh1p and Sdh2p. Other unrelated mitochondrial proteins such as Hsp60 or the β-subunit of ATP synthase were absent from the inclusion bodies, suggesting that the overexpression of Tcm62p may specifically trap SDH subunits. These results suggest a direct interaction of Tcm62p with the subunits of the SDH complex.

Tcm62p expression levels are significantly lower than the levels of the SDH subunits (Ghaemmaghami *et al.*, 2003). This lack of stoichiometry indicates that Tcm62p is not part of the SDH holoenzyme but may interact with SDH subunits during assembly as expected of an assembly factor. Although Tcm62p was shown to comigrate with SDH in a complex, the more abundant SDH holoenzyme was also observed migrating separately in a complex of approximately 130-kDa. Interestingly, in the absence of SDH, Tcm62p was observed to migrate in a larger complex of approximately 450-kDa. Tcm62p was also reported in an approximately 850-kDa complex during gel filtration chromatography, similar in size to the Hsp60p complex (Klanner *et al.*, 2000). Sdh2p did not cofractionate with Tcm62p in this experiment and was observed in a complex of approximately 150-kDa, most likely as part of the SDH holoenzyme. Subsequent co-immunoprecipitation experiments revealed that Tcm62p and Hsp60p reside in distinct complexes.

Tcm62p is as a distant member of the Hsp60 family of molecular chaperones with a low but significant sequence identity to proteins such as the yeast Hsp60p (17% sequence identity; 54% sequence similarity) and to the *E. coli* GroEL (16% sequence identity; 54% sequence similarity). This family of proteins plays a fundamental role in mediating protein folding (Hartl, 1996; Ranson *et al.*, 1998). Currently, no homologs of Tcm62p have been identified, suggesting that it may be a yeast specific chaperone. Aside from its role in SDH assembly, Tcm62p was shown to have additional roles in mitochondrial biogenesis. Tcm62p is required for respiratory competence and mitochondrial protein synthesis at high temperatures (Klanner *et al.*, 2000). Cells lacking Tcm62p cannot grow at elevated temperatures and show decreased levels of mitochondrially translated proteins. Thus, Tcm62p is a member of the molecular chaperones involved in the heat stress response. Tcm62p also acts as a regulator of apoptosis by inhibiting programmed cell death after growth factor withdrawal when heterologously expressed in mammalian cells, although the mechanism of its action is unclear (Vander Heiden *et al.*, 2002). These results suggest that Tcm62p may have additional functions besides its role in the assembly of SDH.

Tcm62p is a 572 amino acid mitochondrial protein with a cleavable N-terminal presequence (Dibrov *et al.*, 1998). Hydropathy analysis suggested that it is an integral membrane protein with a single transmembrane domain. Its transmembrane topology was demonstrated by the accessibility of the C-terminus in the intermembrane space to proteinases and the cleavage of the N-terminal presequence in the mitochondrial matrix. Tcm62p is resistant to solubilization from mitochondrial membranes using high concentrations of salt and urea. However, Tcm62p only shows a partial resistance to solubilization by carbonate extraction at pH 10.5. Resistance to carbonate extraction is a strong indication that a protein is an integral membrane protein. Based on protease accessibility studies, Klanner *et al.* suggested that Tcm62p is a soluble protein of the mitochondrial matrix. It is possible the C-terminal epitope tagging used in the initial characterization of Tcm62p may account for the differing results.

In this chapter, I describe my studies of the structure and function of Tcm62p and how they provide insight into the biogenesis of SDH. It is essential that we understand MRC assembly and biogenesis, not only in context of biological energy metabolism but also because of its emerging importance in human disease (Borisov, 2002; DiMauro and Schon, 2003; DiMauro, 2004). The high degree of structural and functional conservation of SDH in eukaryotes ensures that our yeast studies are likely to be pertinent to human biology. Human diseases resulting from defects in SDH assembly proteins have been proposed, highlighting the need for further studies in this area (Parfait *et al.*, 2000).

As a distant member of the Hsp60 family of proteins, Tcm62p is potentially capable of ATP hydrolysis. Using site-directed mutagenesis, we examined several residues present in conserved motifs known to be involved in ATP binding and hydrolysis. However, the mutations we generated did not affect function, suggesting that Tcm62p is not an ATPase. Previous studies have shown a discrepancy in the physical properties ascribed to Tcm62p. I revisited this issue by investigating the detergent solubility of Tcm62p. We generated polyclonal antibodies towards Tcm62p and alleviated the need for epitope tagging. Our results show that non-ionic detergents are required to release Tcm62p from the membrane, strongly suggesting that it is an integral membrane protein. Tcm62p is partially solubilized by carbonate extraction. I examined the two residues in the putative transmembrane domain, Glu-486 and Lys-490 that might contribute to the extractability of the protein. Mutation of these residues to alanine increased the resistance of Tcm62p to carbonate extraction but did not fully prevent it. This indicates that the hydrophobic interactions between Tcm62p and the membrane bilayer may not be particularly strong.

Previous investigations have revealed that Tcm62p is present in various high molecular weight complexes. We revisited this issue regarding the oligomeric state of the native Tcm62p complexes using BN-PAGE. Tcm62p exists predominantly as a ~450-kDa complex. However, another high molecular weight complex was also seen migrating at approximately 550-kDa. These complexes are stable and their abundance does not change with the expression of SDH subunits. Using an affinity pull down approach, I showed that the Tcm62p complexes are not homo-oligomeric but rather contain additional proteins. Purification of the Tcm62p complexes confirmed additional proteins are present in the complexes. These results strongly suggest that additional proteins are likely involved in the biogenesis of SDH.

2.2 Materials and Methods

Yeast strains, media, culture conditions and the isolation of mitochondria – The yeast strain used in this study, *Δtcm62* (YPH499, *Δtcm62::HIS3*) has been described previously (Dibrov *et al.*, 1998). Yeast strains were grown at 30 °C using the following media: YPD (1% yeast extract, 2% peptone, 2% glucose), YPG (1% yeast extract, 2% peptone 3% glycerol), SD (0.67% yeast nitrogen base, 2% glucose) and SG (0.67% yeast nitrogen base, 3% glycerol) (Oyedotun and Lemire, 1997). For the isolation of mitochondria, cells were cultured in lactate medium (0.3% yeast extract, 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 0.06% $MgSO_4{\cdot}7H_2O$, 0.05% $CaCl_2$, 0.003% $FeCl_3$, 2% lactate) at 30 °C and prepared as described (Daum *et al.*, 1982).

Plasmids and cloning – The plasmids pTCM62-7 and pTCM62-myc have been previously described (Dibrov *et al.*, 1998). pTCM62-7 is the low copy *TRP1*-selectable vector pRS414 (Stratagene) containing the wild-type *TCM62* gene. pTCM62-myc is the same plasmid but encodes a modified version of Tcm62p with the c-*myc* epitope at the C-terminus. The plasmids pTCM62-28 and pTCM62-29 were constructed by cloning a 1.8-kb *Xho* I-*Sst* I fragment from pTCM62-7 and pTCM62-myc respectively, into the low copy *LEU2*-selectable vector pRS415 (Stratagene). The plasmids pDEST15-NT and pDEST15-CT were generated for the production of antibodies using Gateway Cloning technology (Invitrogen). pDEST15-NT encodes a fusion protein consisting of glutathione *S*-transferase and residues 1-468 of Tcm62p, while pDEST15-CT is a similar fusion construct comprising residues 492-572 of Tcm62p. Site-directed mutants were generated using a megaprimer mutagenesis method and confirmed by sequencing the entire gene as described (Sarkar and Sommer, 1990).

Purification of Tcm62p – To facilitate the isolation of Tcm62p, the *Δtcm62* strain was transformed with pTCM62-His. This plasmid is the same as pTCM62-7 but encodes a C-terminal hexahistidine-tagged version of Tcm62p. Mitochondria isolated from this strain were resuspended at a concentration of 1 mg/mL in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 5 mM imidazole) containing 2.5 mM phenylmethylsulfonyl fluoride, Complete EDTA-free protease inhibitor cocktail tablets (Roche) and 1% digitonin. After gentle mixing for 60 min at 4 °C, the sample was clarified by centrifugation at 100,000 × *g* for 30 min at 4 °C. The cleared lysate was mixed gently for 2 hours with Ni-NTA-agarose beads (Qiagen). The slurry was poured into a column, washed with lysis buffer containing 12.5 mM imidazole and eluted with lysis buffer containing 300 mM imidazole. Both wash and elution buffers contained digitonin at a final concentration of 0.05 %. The eluate was dialyzed against 50 mM NaH_2PO_4, pH 8.0, 100 mM NaCl, 20% glycerol [v/v]) overnight at 4 °C using Elutatube Maxi vials (Fermentas).

Electrophoresis and Western blot analyses – Samples were solubilized in gel loading buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Proteins were transferred electrophoretically to nitrocellulose membranes as described by the manufacturer (Tyler Research Instruments). Blots were treated with rabbit polyclonal antisera against Atp2p, Sdh2p (Dibrov *et al.*, 1998) and Tcm62p. Mouse monoclonal antisera against the c-*myc* (Millipore) and hexahistidine

(Qiagen) epitopes were also used. Detection of antigens was with peroxidase-labelled goat anti-rabbit or goat anti-mouse secondary antibodies and the Enhanced Chemiluminescence Western Blotting System (GE Life Sciences).

Blue native gel electrophoresis – Mitochondria (100 ug) were solubilized in extraction buffer (1.75 M amino-caproic acid, 50 mM Tris HCl, 75 mM Bis–Tris, pH 7.0) at a protein concentration of 2 mg/mL. A variety of non-ionic detergents were added to a final concentration of 1%. After incubation at 4 °C for 15 minutes, samples were centrifuged at 14,000 × *g* for 25 min. 2 μL of sample buffer (5% Serva Blue G in 0.5 M amino-caproic acid) were added to the supernatant and gently mixed. For chromatographically purified proteins, samples were loaded directly onto gels without the addition of sample buffer. Samples were analyzed on 5-8% gradient blue native gels as previously described (Schägger and von Jagow, 1991) except that electrophoresis was done at 4 °C between 40 and 60 volts for 2 hr and followed by overnight (12 to 16 hr) at 150 volts in buffer lacking Serva Blue G.

Gels used for subsequent SDS-PAGE analysis were dissected and the gel slices were incubated in 1% SDS (w/v), 1% 2-mercaptoethanol (v/v) solution for 1 hr at room temperature. Proteins were electroeluted from gel slices using Elutatube Midi vials (Fermentas) according to manufacturer's instructions at 4 °C and trichloroacetic acid (TCA) precipitated for SDS-PAGE analysis.

Gels used for western blot analysis were incubated in 20 mM Tris-base, 150 mM glycine, 20% methanol (v/v), 0.08% SDS (w/v) for 10 min before electrophoretic transfer to Immobilon-P polyvinylidene fluoride membranes (Millipore) (Dekker *et al.*, 1997).

Following the electrophoretic transfer, excess Serva Blue G stain was removed by incubating the membranes for 30 min at 50 °C in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS [w/v]) (Nijtmans *et al.*, 2002). Membranes were subsequently washed several times with Tris buffered saline to remove residual 2-mercaptoethanol and prepared for Western blotting.

Antibody production and affinity purification – The generation and purification of polyclonal antisera against Tcm62p were performed following previously published protocols (Harlow and Lane, 1998). Fusion proteins comprising glutathione *S*-transferase and the N- or C-termini of Tcm62p were expressed in *E. coli* strain BL21-SI (Invitrogen). Following expression in *E. coli*, the fusion proteins were isolated as inclusion bodies (Koerner *et al.*, 1991), purified by electroelution and injected into rabbits without any additional processing.

For the affinity purification of the antibodies, full length Tcm62p containing a hexahistidine tag was expressed in the *E. coli* strain BL21-SI (Invitrogen) and isolated as inclusion bodies (Koerner *et al.*, 1991). The protein was solubilized and purified on a Ni-NTA column (Qiagen) under denaturing conditions according to manufacturer's instructions. The purified protein was TCA precipitated, resolved by SDS-PAGE and transferred to nitrocellulose. After staining with Ponceau S stain, strips containing the antigen band were cut out and blocked overnight in Tris buffered saline containing 10% milk powder. Strips were incubated with antiserum for several hours and subsequently washed 5 times with Tris buffered saline. Antibody was eluted with 0.2 M glycine, 1mM

EDTA, pH 2.8 and neutralized with 0.2 volumes of 1M Tris-HCl, pH 8.0 and 0.1 volumes of 10X Tris buffered saline.

Miscellaneous methods - Digitonin was recrystallized according to the manufacturer's protocol (Fluka). Protein quantification was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories) using bovine serum albumin as the standard. *E. coli* transformations and recombinant DNA methods have been described (Sambrook *et al.*, 1989; Bullis and Lemire, 1994). Plasmids were introduced into the yeast strains by lithium acetate-mediated transformation (Gietz *et al.*, 1992). For visualization of proteins, gels were stained with the Silver Quest Silver Staining Kit according to manufacturer's instructions (Invitrogen).

2.3 Results

Mutation of Tcm62p residues does not affect function – As a distant member of the Hsp60 family, it was proposed that Tcm62p might be an ATPase (Dibrov *et al.*, 1998). Chaperones that are actively involved in the assembly process, so called foldases require energy from nucleotide hydrolysis for productive folding (Ranson *et al.*, 1998). Alignment of Tcm62p and Hsp60 sequences revealed the presence of several highly conserved motifs important for ATPase function in Tcm62p (Dibrov *et al.*, 1998) (Figure 2.1). One such motif, **DGTTT** (residues 87-91, numbering and sequence are from GroEL; Figure 2.1) is associated with ATP binding and includes residues of the phosphate-binding loop (Braig *et al.*, 1994; Boisvert *et al.*, 1996). Asp-87 in this motif is essential for ATPase activity in GroEL (Fenton *et al.*, 1994). In Tcm62p, the corresponding sequence is **DNLTAT** (residues 121-126). Asp-398 in GroEL is responsible for the coordination of Mg^{2+} upon ligand binding; mutation of this residue to alanine disrupts ATP turnover in GroEL (Rye *et al.*, 1997; Xu *et al.*, 1997). The corresponding residue in Tcm62p is Asn-429. Other motifs near the ATP-binding site include the sequences TIT*X*DG (residues 48-53) and GGG (residues 414-416). The first motif is absent in Tcm62p, while the second is present as GYG (residues 446-448) (Kim *et al.*, 1994). We mutated Asp-129, Asn-429, Gly-446 and Gly-448 to alanine to determine whether any of these residues are important for Tcm62p function. All mutations were generated by site-directed mutagenesis and introduced into the *Δtcm62* strain. Mutant strains were tested for their ability to grow on a non-fermentable carbon source such as glycerol. *Δtcm62* has a slow growth phenotype when grown on glycerol containing medium (Dibrov *et al.*, 1998). All mutants showed growth comparable to the wild-type and were deemed respiration competent (data not shown).

Tcm62p is a membrane protein – Conflicting evidence on whether Tcm62p is an integral membrane protein of the mitochondrial inner membrane has been presented. We re-examined this issue by investigating the detergent solubility of native Tcm62p. To follow Tcm62p solubilization, polyclonal antibodies directed towards the N- and C-termini of Tcm62p were generated. We tested the ability of three non-ionic detergents with differing structures to solubilize Tcm62p from mitochondrial membranes: Thesit ($C_{12}E_9$), a polyoxyethylene detergent, dodecyl-β-D-maltoside (DDM), an alkyl glycoside detergent and digitonin, a steroid glycoside detergent. At a mitochondrial protein concentration of 1 mg/mL, 2% Thesit was required to release the majority of the protein from the membrane (Figure 2.2). The detergents DDM and digitonin solubilized Tcm62p more efficiently with the majority of the protein being solubilized at the detergent concentrations of 1%. While Tcm62p was fully solubilized by 2% DDM, some Tcm62p was not fully extracted with either Thesit or digitonin. Porin, an integral membrane protein of the mitochondrial outer membrane (Forte *et al.*, 1987), was not solubilized under any of the conditions tested.

We also re-examined the attachment of Tcm62p to the membrane by carbonate extraction. Previous results using 0.1 M Na_2CO_3, pH 11.5, completely solubilized Tcm62p, whereas a similar treatment at pH 10.5 was only partially effective at solubilizing the protein (Dibrov *et al.*, 1998). It was hypothesized that the partial solubilization by Na_2CO_3 was due to the presence of charged amino acids (Glu-486 and

Lys-490) in the only potential transmembrane segment in Tcm62p (Figure 2.1). These residues may form a salt bridge compatible with membrane integration at neutral pH but destabilize membrane attachment when only the glutamic acid residue will be charged at pH 11.5. We mutated both residues to alanine and examined the susceptibility of this mutant protein to carbonate extraction. As shown in Figure 2.2, the results indicate that the mutant protein is less extractable than the wild-type. At pH 10.5, approximately half of the mutant protein was retained in the membrane, while the majority of the wild-type protein was extracted. At pH 11.5, a portion of the mutant protein remained in the membrane, whereas the wild-type protein was completely solubilized under these conditions. However, the mutant protein is only partially resistant to carbonate extraction since the majority of the protein is found in the soluble fraction at pH 11.5. Porin, as expected, was not released from the membrane when subjected to carbonate extraction. Based on these results, we suggest that Tcm62p is a membrane protein and that the residues Glu-486 and Lys-490 play a role in the susceptibility of this protein to carbonate extraction.

Tcm62p is part of large complexes required for proper SDH assembly – Previous results indicated that Tcm62p exists in various high molecular weight species. One of the complexes described in our lab was judged to be Tcm62p in association with SDH to form a complex of approximately 200-kDa. In the absence of SDH subunits, Tcm62p migrated in a new complex of approximately 450-kDa. In contrast, gel filtration analysis by Klanner *et al.* revealed Tcm62p to be a component of an even larger complex of approximately 850-kDa. We re-examined the oligomeric state of the native Tcm62p using BN-PAGE, a method suited to separating large membrane bound complexes such as the mitochondrial respiratory chain enzymes (Schägger and von Jagow, 1991). With the generation of polyclonal antibodies towards Tcm62p, we were able to examine the properties of the wild-type protein and remove any problems associated with epitope tagging. Our results indicate that native Tcm62p resides in a large, stable complex of approximately 450-kDa (Figure 2.3). In agreement with previous findings, this complex is present when SDH is not assembled, as in an *SDH2* deletion mutant. However, the 200-kDa complex (Tcm62p associated with SDH) was not observed in these experiments (Figure 2.3). The Tcm62p complex is required for SDH assembly; in the absence of Tcm62p, properly assembled SDH is not detected. This is in agreement with previous results indicating that mitochondria lacking Tcm62p are specifically depleted of SDH subunits (Dibrov *et al.*, 1998).

Tcm62p is a component of several high molecular weight complexes – In the course of analysis of the Tcm62p complexes, we determined that it resides not only in a complex of approximately 450-kDa, but also in higher molecular weight species as well. Tcm62p is also found in a less abundant complex of approximately 550-kDa and two minor species migrating at a molecular weights greater than 800-kDa (Figure 2.4). Based on the sizes of these larger complexes, we hypothesized that they may correspond to dimers of the 450- and 550-kDa species. The 550-kDa complex is sensitive to some detergents as it is not readily observed when DDM is used for solubilization (Figure 2.4B). Triton X-100 does not appear to affect its stability. Interestingly, the 550-kDa complex is stable in the presence of either detergent when Tcm62p is mildly overexpressed. Overexpression of

Tcm62p was achieved by transforming two low copy plasmids with different selectable markers, each containing the *TCM62* gene. Using a metal-affinity chromatography and with BN-PAGE, we are able to isolate the 450- and 550-kDa complexes with fairly high purity as judged by silver staining of native gels (Figure 2.6A). Western blot analysis verified that both bands indeed contain Tcm62p (Figure 2.6B). The migrations of the purified complexes are similar to the migration of complexes solubilized from mitochondria, indicating their stability throughout the purification steps.

Tcm62p interacts with the other proteins to form high molecular weight complexes – The presence of Tcm62p in high molecular weight complexes raises questions as to the nature of their composition. These complexes could be homo-oligomeric, containing multiple copies of Tcm62p. Members of the Hsp60 family of proteins form homo-oligomeric assemblies containing 7 monomers (Hartl, 1996). Alternatively, Tcm62p could interact with other proteins to form these complexes. Another possibility is that the 450-kDa species is homo-oligomeric and the 550-kDa complex is formed by the addition of other proteins. We examined the composition of the Tcm62p complexes using two approaches. The first method was an affinity pull down assay using hexahistidine-tagged Tcm62p co-expressed with a c-*myc*-tagged version of the protein. We assumed that when expressed together, the two tagged versions of Tcm62p would not segregate into distinct populations, but rather would intermix. We predicted that homo-oligomeric complexes should simultaneously contain both c-*myc* and hexahistidine-tagged Tcm62p. Affinity capture of the hexahistidine-tagged Tcm62p using Ni-NTA beads should also recover some c-*myc*-tagged Tcm62p. Solubilized mitochondrial membranes containing both the hexahistidine-tagged and c-*myc*-tagged versions of Tcm62p were incubated with Ni-NTA beads, washed and the bound material was resolved on SDS-PAGE. Solubilized mitochondrial membranes containing the hexahistidine-tagged and wild-type versions of Tcm62p were used as a control. As shown in Figure 2.5A, only the hexahistidine-tagged Tcm62p is captured; the c-*myc*-tagged Tcm62p is absent. We showed that the Ni-NTA beads do not pull down native Tcm62p not specifically; wild-type protein was not recovered in the pellet fraction. These results suggest that the Tcm62p complexes contain only one copy of Tcm62p and that they are not homo-oligomeric in their composition. As a control, we verified that both versions of Tcm62p were being expressed simultaneously and that both were functional. Mitochondria containing various versions of Tcm62p were solubilized and resolved by BN-PAGE. Gel slices containing proteins in 400-600 kDa range were excised; the proteins contained were electroeluted and resolved using SDS-PAGE. The second electrophoretic step was required because we were unable to directly detect the epitope-tagged Tcm62p complexes after BN-PAGE. The use of monoclonal antibodies to detect complexes resolved by BN-PAGE is difficult because the epitopes recognized by the antibodies may be hidden or buried in the complexes (Nijtmans *et al.*, 2002). Western blotting with the corresponding monoclonal antibodies detected the presence of both the c-*myc* and hexahistidine epitopes, indicating that both tagged versions of Tcm62p were expressed simultaneously and assembled into the high molecular weight complexes (Figure 2.5B).

We further examined the compositions of the purified complexes. After metal-affinity chromatography and BN-PAGE, gel slices corresponding to the 450- and 550-kDa complexes were excised and the proteins electroeluted. The electroeluted proteins were resolved on SDS-PAGE and visualized by silver staining. As shown in Figure 2.6C, numerous proteins are present in both isolated complexes. The protein band patterns suggest that these complexes are composed of the same proteins, with the 550-kDa complex containing at least two additional proteins. These results indicate that Tcm62p interacts with many additional proteins to form two stable multiprotein complexes.

2.4 Discussion

To further our understanding of the involvement of chaperones in the assembly of SDH, we investigated the structure and function of Tcm62p. We examined several aspects of this protein in an attempt to understand its role as an assembly factor. Its primary sequence indicates that it is a distant member of the Hsp60 family of molecular chaperones (Dibrov *et al.*, 1998). Hsp60 family members are actively involved in the assembly process of substrate proteins, require energy for productive folding and are often ATPases (Ranson *et al.*, 1998). A sequence alignment of Tcm62p and Hsp60 revealed that sequence motifs important for ATPase function are present in Tcm62p (Figure 2.1) (Dibrov *et al.*, 1998). We mutated several residues thought to be involved in ATP binding and hydrolysis. However, none of the mutations caused a change in the respiratory competence of the tested strains, suggesting that Tcm62p function was not seriously compromised. Although these results do not eliminate the possibility that Tcm62p is an ATPase, they do suggest Tcm62p may employ an ATP-independent mode of action. ATP hydrolysis is not an absolute requirement for chaperone function (Ellis, 1997). Other mitochondrial chaperones, such as the prohibitin complex do not possess ATPase activity. The prohibitin complex does not engage in active folding of substrate proteins but rather acts as a holding complex to prevent aggregation of unassembled polypeptides (Nijtmans *et al.*, 2000; Nijtmans *et al.*, 2002). Tcm62p may perform a similar function or act as a scaffold to facilitate the incorporation of the numerous cofactors into the subunits of SDH. Alternatively, we did not mutate the correct residues to disrupt ATPase function.

Previous studies have led to conflicting conclusions about Tcm62p's physical properties (Dibrov *et al.*, 1998; Klanner *et al.*, 2000). There is conflicting evidence on whether Tcm62p is an integral membrane protein. The protein previously characterized in our lab was tagged at the C-terminus with a hemaglutinin epitope and the tag may have interfered with targeting and/or assembly, resulting in erroneous results. We revisited Tcm62p's membrane attachment by examining the detergent solubility of native Tcm62p. Integral membrane proteins require detergents to extract them from the lipid bilayer and shield exposed hydrophobic transmembrane domains from the aqueous environment (von Jagow and Schägger, 1994). Our results indicate that high concentrations of non-ionic detergents are required to release Tcm62p from the membrane (Figure 2.2A). In agreement with previous findings, treatment with either 0.5 M NaCl or 8 M urea does not extract native Tcm62p from mitochondrial membranes (data not shown). This evidence strongly suggests that Tcm62p is not a peripheral membrane protein but rather is anchored to the membrane by non-ionic interactions.

Although the majority of evidence points to Tcm62p being a membrane protein, Tcm62p is not fully resistant to carbonate extraction. Resistance to carbonate extraction indicates strong hydrophobic interactions with the core of the lipid bilayer and is a property used to classify integral membrane proteins. It was previously suggested that the residues Glu-486 and Lys-490 in the putative transmembrane domain may form a salt bridge compatible with membrane integration at neutral pH but destabilize membrane attachment at elevated pHs when only the glutamate will be charged. We examined the effects on carbonate extraction of mutating both of these charged residues to alanine, a non-polar residue. Our results show that this mutant protein is more resistant to extraction than the wild-type (Figure 2.2B). However, it is still only partially resistant to carbonate extraction and the majority of the protein is recovered in the soluble fraction. Mutation of these residues to a more hydrophobic residue such as leucine may facilitate increased resistance of Tcm62p to this treatment. This would be strong evidence that Tcm62p is integrated into the membrane bilayer with a single transmembrane domain.

Our results indicate that Glu-486 and Lys-490 are only partially responsible for the carbonate extractability of Tcm62p. A few other integral membrane proteins have also been shown to be susceptible to carbonate extraction. These include the single transmembrane domain proteins Mdm38p, Ylh47p and the Rieske Fe-S protein (Rip1p) of complex III of the MRC (Frazier *et al.*, 2006). These results, particularly for Rip1p, were explained by the close proximity of the single transmembrane helix to other proteins rather than it being completely embedded in the lipid bilayer (Lange and Hunte, 2002), making it more sensitive to carbonate extraction than conventional membrane-spanning proteins. We believe that this is also the situation for Tcm62p. In summary, our evidence suggests that Tcm62p is an integral membrane protein.

Tcm62p has previously been detected in several oligomeric assemblies. Our lab showed that Tcm62p forms a complex with SDH and in the absence of SDH, Tcm62p was present as a 450-kDa species (Dibrov *et al.*, 1998). Klanner *et al.* also observed Tcm62p in an 850-kDa complex. Therefore, we re-examined the oligomeric forms of Tcm62p in mitochondrial membranes. Our evidence shows that it is part of two high molecular complexes migrating at approximately 450-kDa and 550-kDa (Figures 2.3 and 2.4). The 450-kDa complex is the more abundant species and its presence is consistent with the previously observed oligomeric form. The 550-kDa complex is sensitive to detergents such as DDM suggesting that its stability is dependent on hydrophobic interactions between the subunits (Figure 2.4B). Interestingly, the 550-kDa complex is stable when Tcm62p is mildly overexpressed and its abundance is significantly higher when the complexes are isolated chromatographically (Figures 2.4B and 2.6A).

In contrast to the previous results, the 450-kDa complex is stable and its formation is independent of the expression of the SDH subunits (Figure 2.3B). We did not observe a 200-kDa complex, previously postulated to be SDH in association with Tcm62p. The 200-kDa complex was detected using hemagglutinin-tagged protein; the tag may have altered the affinity of Tcm62p for the subunits of SDH. Alternatively, our solubilization conditions may have disrupted the non-covalent interactions between Tcm62p and the assembled SDH holoenzyme. In addition to the 550-kDa complex, we also detected two higher molecular weight species migrating above 800-kDa (Figure 2.4). We suggest that these species may be dimers of the 450- and 550-kDa complexes. These complexes may be the 850-kDa species observed in the gel filtration experiments of Klanner *et al*. The higher abundance of the smaller complexes in our experiments may be attributable to the detergents used. Klanner *et al.* only observed the 850-kDa species while using a much lower concentration of detergent (0.1% Triton X-100) to extract Tcm62p from the mitochondria. Our use of a higher detergent concentration (1%) to extract Tcm62p from mitochondrial membranes may be disrupting the weak interactions between dimeric complexes resulting in the much lower abundance of the large complexes and higher levels of the smaller complexes. This would also suggest that the dimerization interactions may be hydrophobic in nature, because they can be disrupted by detergents. The relationship of the different complexes to SDH assembly remains completely unknown.

The composition of these high molecular weight complexes is of great interest. Based on the sequence similarities between Tcm62p and chaperonins, it was proposed that the Tcm62p might form a homo-oligomeric structure composed of seven monomers (Dibrov *et al.*, 1998). Our results suggest that the Tcm62p complexes only contain one copy of Tcm62p per complex (Figure 2.5). The 450- and 550-kDa complexes are hetero-oligomeric in their composition and contain many proteins, several of which seem to be shared by the two complexes (Figure 2.6C). The 550-kDa complex contains at least two additional proteins. If both complexes participate in SDH assembly and all their protein components are necessary for SDH assembly, then we can conclude that many other loci involved in SDH biogenesis remain to be discovered. Our attempts to identify the proteins in the complexes by mass spectrometry have been unsuccessful so far. This is in part due to the low abundance of the complexes (Ghaemmaghami *et al.*, 2003). Identifying the proteins that form the Tcm62p complexes will be essential to furthering our understanding of SDH biogenesis.

In conclusion, we have provided evidence that Tcm62p is not an ATPase, although it contains sequence motifs found in ATPases of the Hsp60 family of proteins. Our evidence suggests that Tcm62p is an integral membrane protein, although the hydrophobic interactions that anchor into the membrane are weak. The presence of two charged residues in the transmembrane domain suggests the transmembrane segment may be involved in electrostatic interactions with other membrane proteins in the Tcm62p complexes. We have examined the oligomeric forms of Tcm62p and show that it forms four high molecular weight species. The two smaller, more abundant complexes are not homo-oligomeric. We conclude that much remains to be discovered about the biogenesis of the yeast SDH.

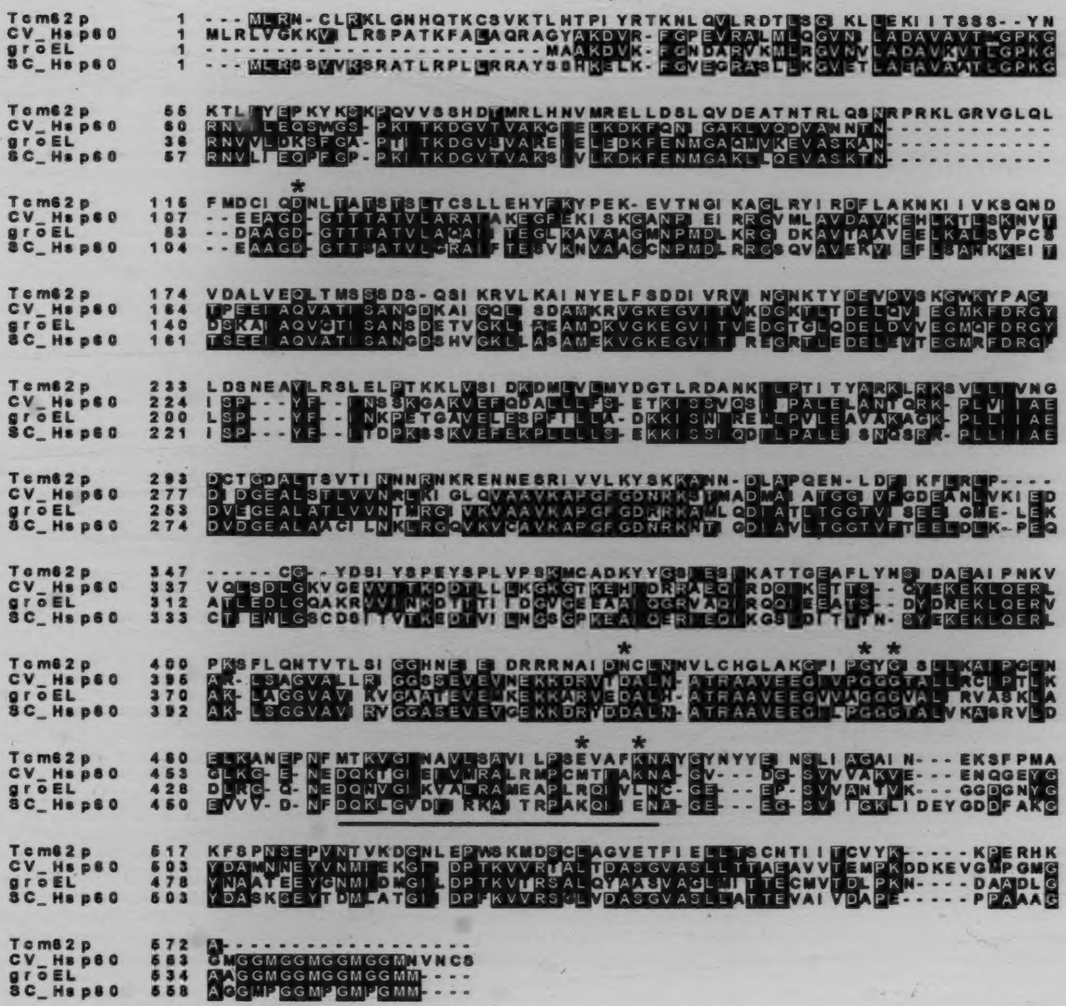

Figure 2.1 Alignment of Tcm62p with chaperonins. The primary sequences of Tcm62p, *C. variipennis* hsp60, *E. coli* hsp60 (GroEL) and the *S. cerevisiae* hsp60 are shown in a multiple sequence alignment. Identical residues are highlighted in black and similar residues are highlighted in grey. Residues examined in this study are indicated with asterisks. The underlined portion is the putative transmembrane domain in Tcm62p. The alignments were generated using Clustal X (Thompson *et al.*, 1997) and Boxshade 3.21.

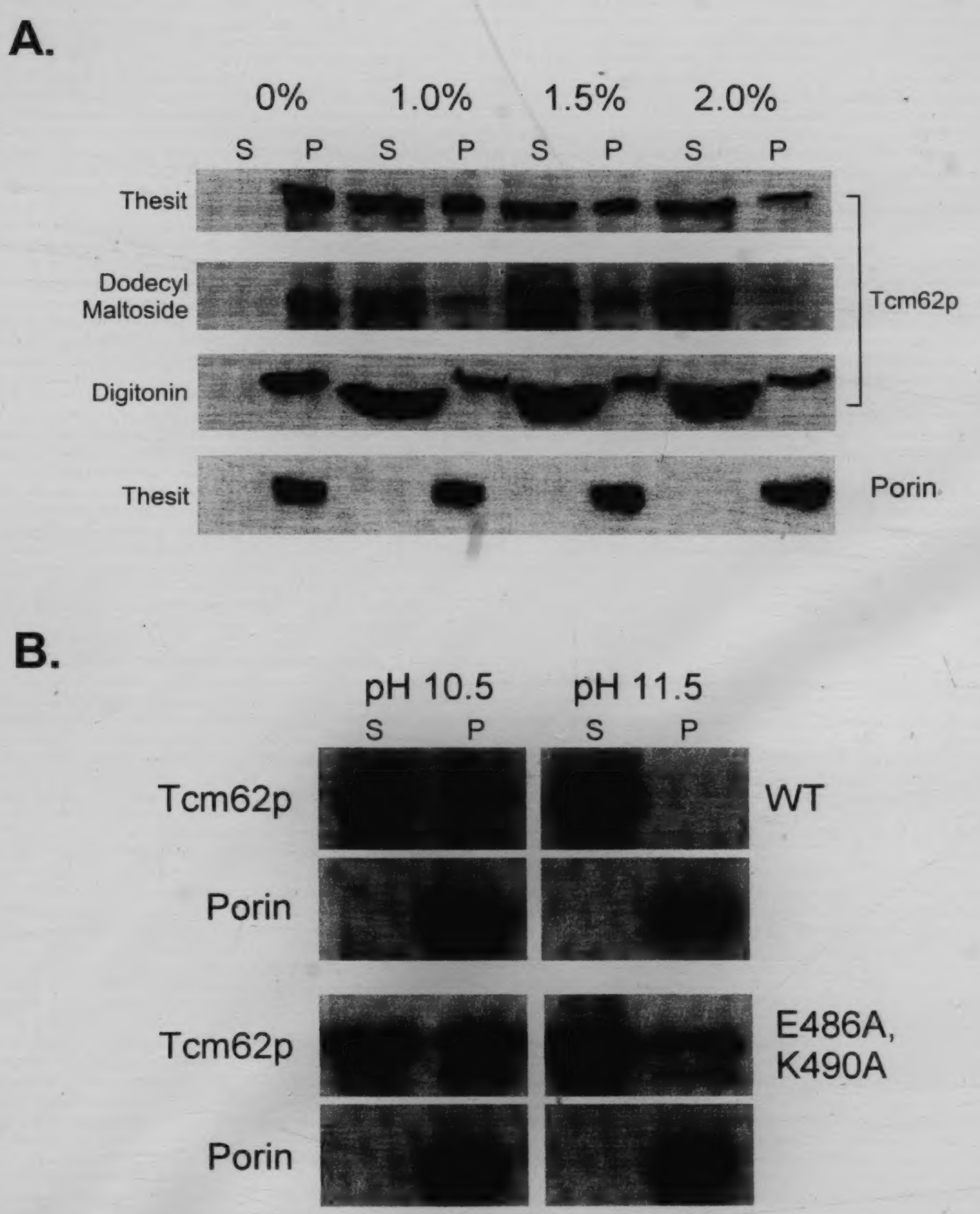
A.
0%
1.0%
1.5%
2.0%
S
P
S
P
S
P
S
P
Thesit
Dodecyl Maltoside
Digitonin
Thesit
Tcm62p
Porin
B.
pH 10.5
pH 11.5
S
P
S
P
Tcm62p
Porin
WT
Tcm62p
Porin
E486A, K490A

Figure 2.2 Tcm62p is a membrane protein. *A*, Mitochondria were solubilized in buffer containing non-ionic detergents at the concentrations indicated and centrifuged at 100,000 × *g* for 30 min at 4 °C. The supernatant (S) and pellet (P) fractions were analyzed by Western blotting using antibodies targeted directed against Tcm62p or porin. *B*, Mitochondria containing either wild-type Tcm62p (WT) or the E486A, K490A mutant Tcm62p were incubated with 0.1 M Na_2CO_3 at either pH 10.5 or 11.5 for 30 min at 4 °C. The samples were fractionated and analyzed in the same manner as in *A*.

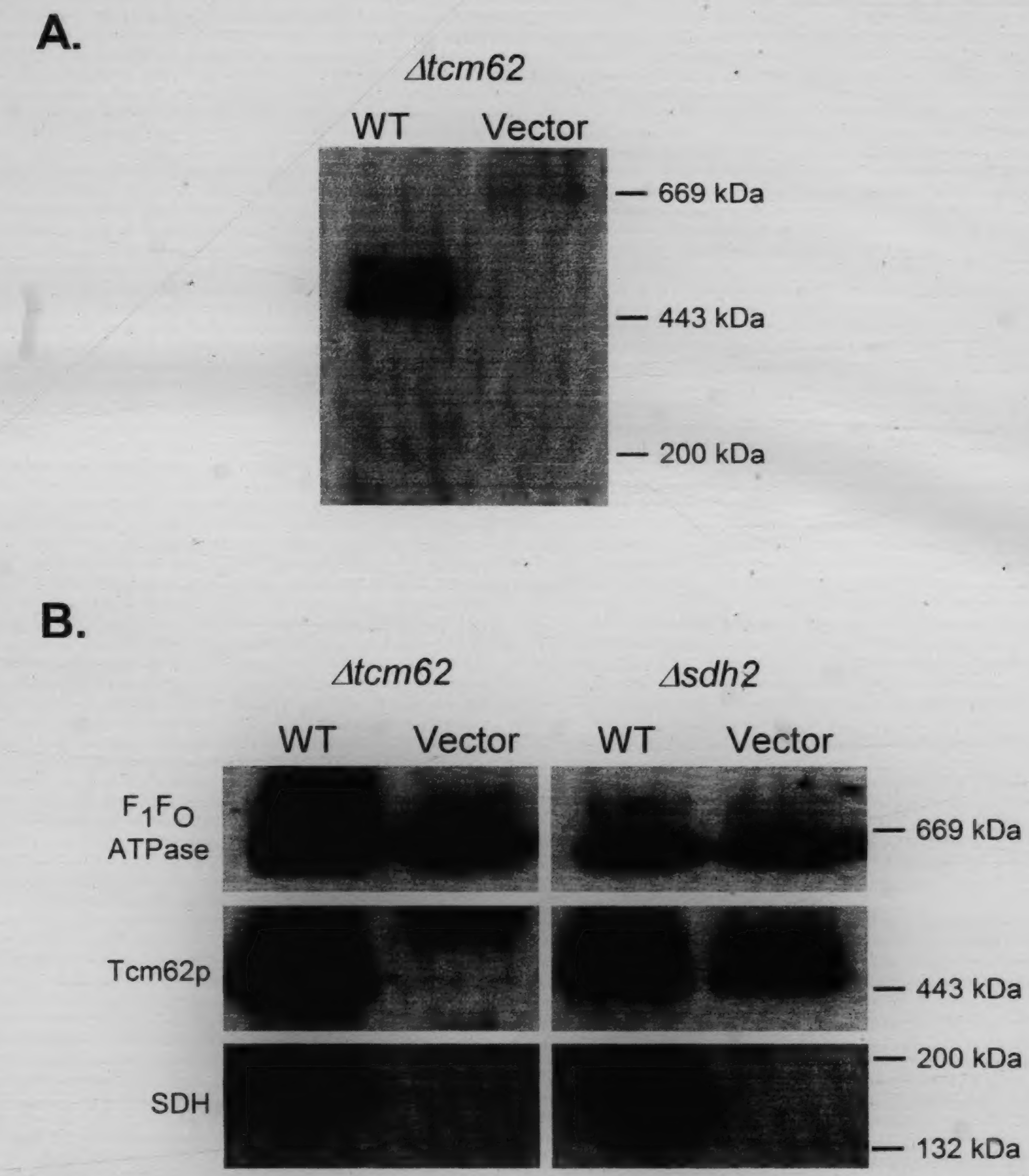
A.
Δtcm62
WT
Vector
669 kDa
443 kDa
200 kDa
B.
Δtcm62
Δsdh2
WT
Vector
WT
Vector
F_1F_O ATPase
Tcm62p
SDH
669 kDa
443 kDa
200 kDa
132 kDa

Figure 2.3 Tcm62p forms stable high molecular weight complexes required for SDH assembly. *A*, Mitochondria isolated from the *Δtcm62* strain transformed with a plasmid containing *TCM62* (WT) or the empty vector were solubilized in buffer containing 1% DDM and proteins were resolved by BN-PAGE. The Tcm62p complex was visualized by Western blot analysis. *B*, Mitochondria isolated from the *Δsdh2* strain transformed with a plasmid containing *SDH2* or the empty vector as well as the mitochondria used in *A* were solubilized in buffer containing 1% DDM. Protein complexes were resolved by BN-PAGE and the Tcm62p, SDH and F_1F_O-ATPase complexes were visualized by Western blot analysis.

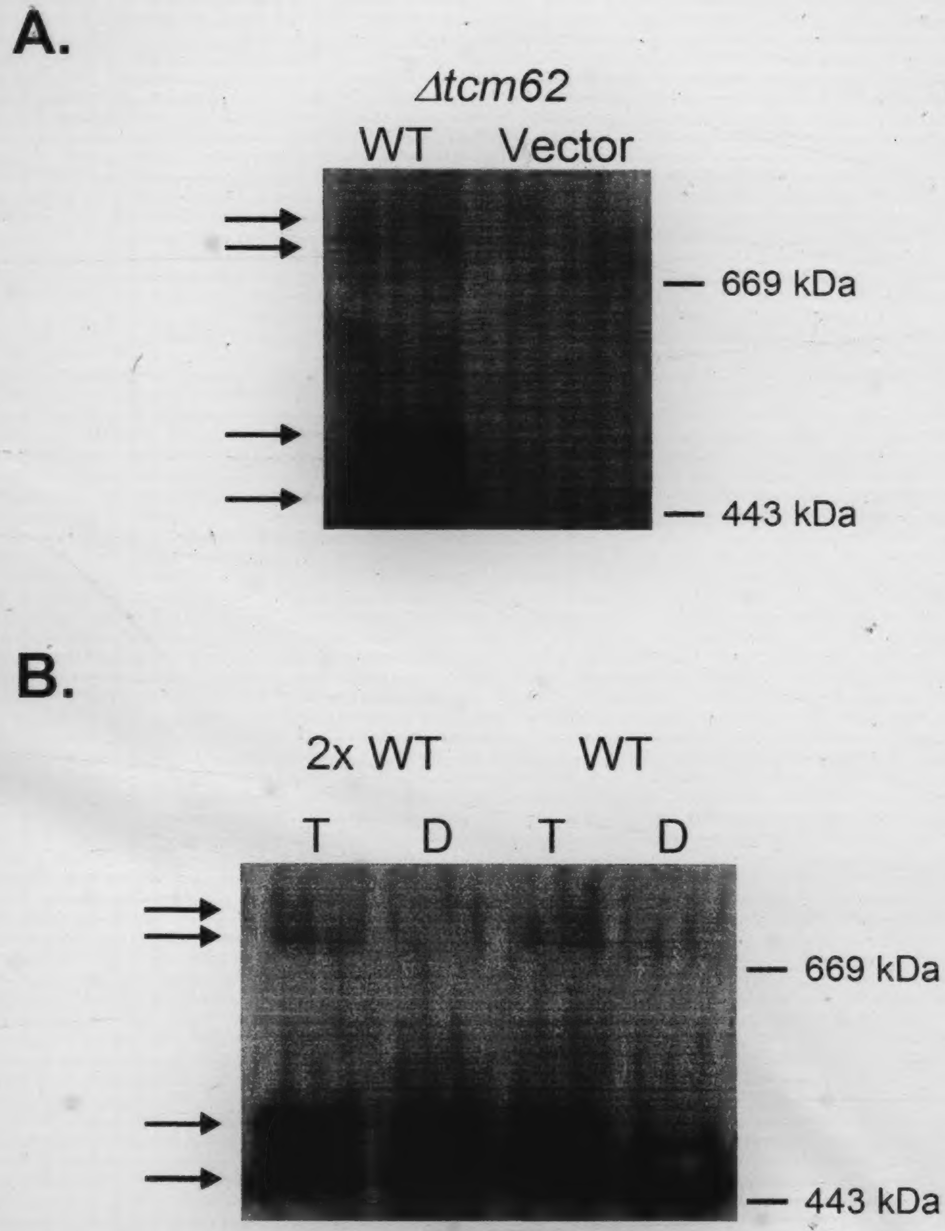

Figure 2.4 Tcm62p is part of several high molecular weight complexes. *A*, Mitochondria isolated from the *Δtcm62* strain transformed with a plasmid containing *TCM62* or an empty vector were solubilized in buffer containing 1% DDM and protein complexes were resolved by BN-PAGE. Tcm62p complexes were visualized by Western blot analysis. *B*, Mitochondria isolated from the *Δtcm62* strain transformed with a single or with two plasmids containing *TCM62* were solubilized with either 1% DDM (D) or Triton X-100 (T). Protein complexes were resolved by BN-PAGE and the Tcm62p complexes were visualized by Western blot analysis.

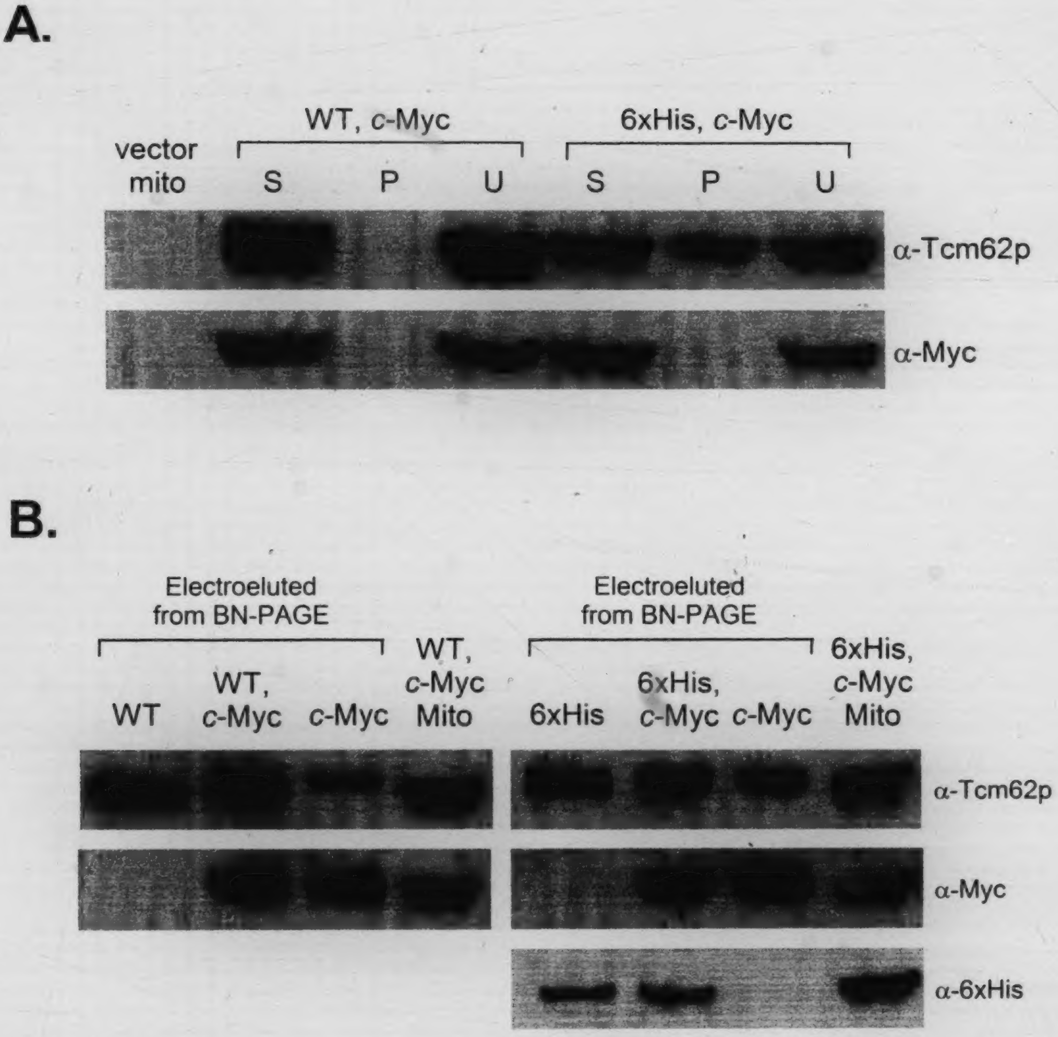
A.
vector mito
WT, c-Myc
S
P
U
6xHis, c-Myc
S
P
U
α-Tcm62p
α-Myc
B.
Electroeluted from BN-PAGE
WT
WT, c-Myc
c-Myc
WT, c-Myc Mito
Electroeluted from BN-PAGE
6xHis
6xHis, c-Myc
c-Myc
6xHis, c-Myc Mito
α-Tcm62p
α-Myc
α-6xHis

Figure 2.5 The high molecular weight complexes contain only one copy of Tcm62p. *A*, Mitochondria containing wild-type (WT) and *c*-myc-tagged or hexahistidine-(6xHis) and *c*-myc-tagged versions of Tcm62p were solubilized in buffer containing 1% digitonin. The lysates were incubated with Ni-NTA beads, subjected to centrifugation and the supernatants containing unbound proteins were removed (S). The pelleted beads were washed with buffer and bound proteins (P) were eluted with sample buffer. Both fractions were resolved by SDS-PAGE and visualized by Western blotting. Untreated lysates (U) and mitochondria lacking Tcm62p were used as controls. *B*, Mitochondria containing various versions of Tcm62p were solubilized in buffer containing 1% digitonin and protein complexes resolved by BN-PAGE. Protein complexes migrating in the range of 400-600 kDa were electroeluted, resolved on SDS-PAGE and visualized by Western blotting. Mitochondria examined in *A* were used as controls for this experiment.

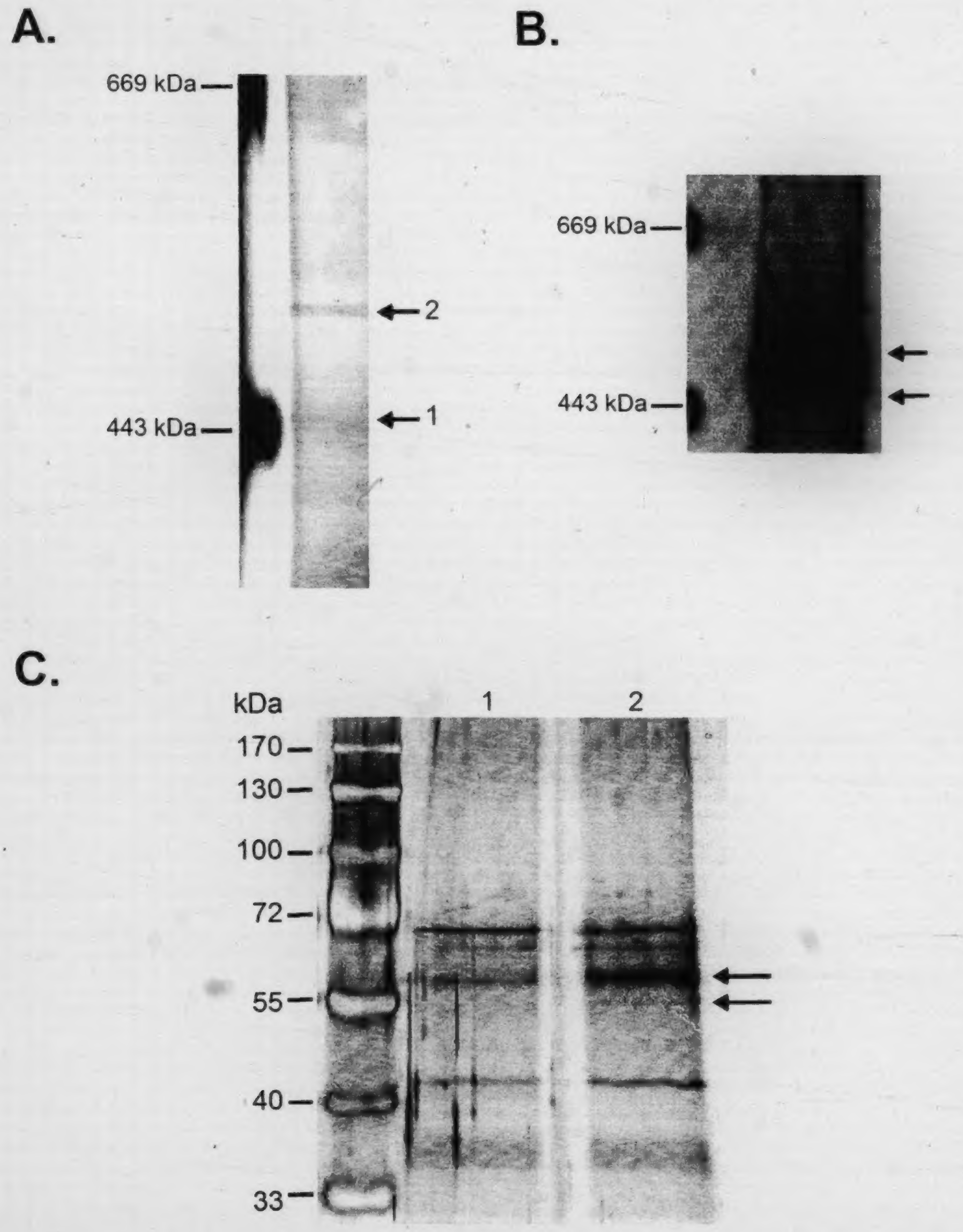
A.
669 kDa
443 kDa
2
1
B.
669 kDa
443 kDa
C.
kDa
1
2
170
130
100
72
55
40
33

Figure 2.6 The purified Tcm62p complexes contain multiple proteins. *A*, Mitochondria containing hexahistidine-tagged Tcm62p were solubilized in lysis buffer containing 1% digitonin and subjected to metal-affinity chromatography. Bound proteins were eluted, subjected to BN-PAGE and visualized by silver stain. *B*, After separation by BN-PAGE as in *A*, Tcm62p complexes were analyzed by Western blot analysis. *C*, Complexes identified in *A* were electroeluted, analyzed by SDS-PAGE and visualized by silver staining. Arrows indicates the bands of interest.

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CHAPTER 3

Tcm62p and the Prohibitin Complex are *Saccharomyces cerevisiae* Mitochondrial Chaperones that Function Independently

3.1. Introduction

Mitochondria play a central role in apoptosis and are involved in many facets of programmed cell death (Raha and Robinson, 2001). The involvement of mitochondria in apoptosis is regulated by the Bcl-2 family of proteins. Depending on the interaction of the particular Bcl-2 protein with the mitochondrion, the effect can be either pro- or anti-apoptotic (Harris and Thompson, 2000; Martinou and Green, 2001). Bcl-2 proteins act by regulating the release of intermembrane space proteins such as cytochrome *c* and apoptosis-inducing factor (AIF) from the mitochondrion (Wei *et al.*, 2000; Martinou and Green, 2001). It has also been proposed that Bcl-2 proteins modulate cell survival pathways through their effects on mitochondrial physiology and metabolism (Vander Heiden and Thompson, 1999). A pro-apoptotic member such as Bax has been shown to inhibit oxidative phosphorylation (Harris *et al.*, 2000) while the anti-apoptotic protein Bcl-x_L allows cells to maintain oxidative phosphorylation even when glycolysis is shut down, as is the case of cells undergoing apoptosis (Vander Heiden *et al.*, 2000; Vander Heiden *et al.*, 2001). It is thought that these proteins may regulate the flow of metabolites across the outer membrane and this represents an evolutionarily conserved function of this family of proteins. All eukaryotic cells experience situations when nutrients are limiting and must have developed mechanisms to maintain metabolite transport across the outer membrane during periods of cellular stress and nutrient deprivation. Although Bcl-2 proteins only exist in metazoan organisms, previous studies show some family members such as Bax, Bak and Bcl-x_L retain at least some of their functions when expressed in yeast (Zha *et al.*, 1996; Jurgensmeier *et al.*, 1997; Minn *et al.*, 1999). This suggests that these proteins function in a conserved pathway to regulate certain aspects of mitochondrial biology common to both yeast and higher eukaryotes.

Vander Heiden *et al.* examined whether the biochemical functions that Bcl-x_L possesses were retained when heterologously expressed in yeast. Given that Bcl-x_L appears to facilitate a switch from glycolytic to oxidative metabolism in mammalian cells, their study examined whether Bcl-x_L could complement yeast mutants defective in their ability to undergo a diauxic shift (Vander Heiden *et al.*, 2002). Among the several genes that were identified, of particular interest was the gene *TCM62* (Vander Heiden *et al.*, 2002). As an assembly factor for SDH, Tcm62p would facilitate a diauxic shift by mediating the assembly of a component of the mitochondrial respiratory chain (Dibrov *et al.*, 1998). This finding reinforces the conserved role of Bcl-x_L in this metabolic process but also suggests that mitochondrial chaperones such as Tcm62p may play some part in apoptosis. Assembly factors for SDH may be of particular importance in light of the fact that this enzyme has been implicated as a sensor for apoptosis induction (Albayrak *et al.*, 2003). In agreement with this hypothesis, it was shown that heterologous expression of Tcm62p in mammalian cells conferred resistance to cell death following growth factor withdrawal (Vander Heiden *et al.*, 2002). In addition, Vander Heiden *et al.* examined the role of a functionally analogous mitochondrial chaperone, the prohibitin complex, and it produced a similar effect as Tcm62p. This finding implicates mitochondrial chaperones as potential regulators of apoptosis as well.

The prohibitin complex acts as a chaperone for MRC complex assembly (Nijtmans *et al.*, 2000; Osman *et al.*, 2007). The two prohibitin proteins, Phb1p and Phb2p form a large molecular weight complex in the mitochondrial inner membrane, which binds to newly synthesized mitochondrial translation products and protects them from degradation by membrane-bound AAA-metalloproteinases (Steglich *et al.*, 1999; Tatsuta *et al.*, 2005). In addition, this complex was recently shown to play an important role in the assembly of the ATP synthase by interacting with its assembly factors, Atp10p and Atp23p (Osman *et al.*, 2007). The prohibitin genes were initially cloned as tumor suppressor genes and deletion of the *PHB* genes in *S. cerevisiae* decreases its replicative life span, but has little effect on its growth otherwise (Dell'Orco *et al.*, 1996; Coates *et al.*, 1997; Nijtmans *et al.*, 2002). Deletion of the *PHB* genes is lethal when combined with mutations of the mitochondrial inheritance machinery (Berger and Yaffe, 1998), the AAA-mitochondrial proteinases (Steglich *et al.*, 1999) or the phosphatidylethanolamine biosynthetic machinery (Birner *et al.*, 2003). The lack of a clear growth phenotype in yeast *PHB* mutants is believed to reflect functional redundancy with other chaperones or assembly factors.

The observation that Tcm62p and prohibitins were shown to act as regulators of apoptosis by inhibiting mammalian cell death after growth factor withdrawal suggested a potential connection between the two chaperones. Both Tcm62p and prohibitins exist as high molecular weight complexes in the mitochondrial inner membrane, function as molecular chaperones and play vital roles in situations of metabolic stress. In light of the previous evidence, we formulated the hypothesis that Tcm62p and prohibitins are functionally analogous or redundant.

In this chapter, we examined whether the two mitochondrial chaperones Tcm62p and the prohibitin complex have overlapping functions. Overexpression of the prohibitin proteins, under both low and high copy conditions, in a Δ*tcm62* background does not suppress its slow growth phenotype. Furthermore, a Δ*phb1*, Δ*phb2*, Δ*tcm62* triple knockout strain has growth properties similar to the single Δ*tcm62* mutant. We conclude based on these experiments that Tcm62p and the prohibitin complex operate in distinct non-overlapping pathways under normal growth conditions.

3.2. Materials and Methods

Yeast strains - The yeast strains used in this study, *Δtcm62* and *Δphb1, Δphb2* have been described previously (Dibrov *et al.*, 1998; Nijtmans *et al.*, 2000). The triple knock out strain, *Δphb1, Δphb2, Δtcm62* is a derivative of *Δphb1, Δphb2* in which the *TCM62* gene was disrupted by homologous recombination. The disruption cassette was created by replacement of a 0.23-kb *Stu*I-*Mun*I fragment in the *TCM62* gene with a 1.1-kb *URA3* gene cassette. In addition, a 0.5-kb *Nsi*I fragment downstream of the cassette insertion site was removed. The disruption cassette was amplified by PCR, transformed into the *Δphb1, Δphb2* strain and uracil prototrophs were selected. Disruption of the *TCM62* gene was confirmed by PCR and by complementation analysis.

Protein isolation and Western blotting - Whole cell extracts were prepared by alkaline lysis (0.25M NaOH, 1% 2-mercaptoethanol) of yeast cells followed by precipitation with trichloroacetic acid (Yaffe and Schatz, 1984). Lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose. Blots were treated with rabbit polyclonal antisera against Por1p and Phb1p (Dibrov *et al.*, 1998; Nijtmans *et al.*, 2000). Detection of antigens was done with peroxidase-labelled goat anti-rabbit secondary antibodies and the Enhanced Chemiluminescence Western Blotting System (GE Life Sciences).

Miscellaneous methods - *E. coli* transformations and recombinant DNA methods have been described (Sambrook *et al.*, 1989; Bullis and Lemire, 1994). Plasmids were introduced into the yeast strains by lithium acetate-mediated transformation (Gietz *et al.*, 1992). The yeast media SD, SG, YPG, and YPGal have been described (Oyedotun and Lemire, 1997). The plasmid pTCM62-7 has been previously described (Dibrov *et al.*, 1998).

3.3 Results and Discussion

Overexpression of a protein can often compensate for the absence of another if the proteins have similar functions. We overexpressed the prohibitin chaperone proteins in a Δ*tcm62* background. Since the prohibitins have a broader range of substrates than Tcm62p, we reasoned that they might be able to function in SDH assembly and restore efficient growth on non-fermentable carbon sources. Deletion of the *TCM62* gene results in slow growth on a rich medium with glycerol as a non-fermentable carbon source (YPG) and no growth on a minimal glycerol medium (SG) (Dibrov *et al.*, 1998). Both centromeric (1-2 copies per cell) and multi-copy plasmids encoding the *PHB1* and *PHB2* genes were transformed into the Δ*tcm62* strain. No observable differences in the growth rates of the Δ*tcm62* strain transformed with empty vector controls or with the *PHB1* and *PHB2* containing plasmids were detected (data not shown). To ensure that expression of the prohibitin proteins was occurring, whole cell extracts were isolated and subjected to immunoblotting with anti-Phb1p antiserum. Western blot analysis revealed that both low and multi-copy plasmids led to the overexpression of Phb1p in the *Δtcm62* strain (Figure 3.1). The levels of the Phb1p and Phb2p are interdependent; overexpression of either gene alone does not result in additional complex formation and loss of one protein leads to the disappearance of the other. Only when the *PHB1* and *PHB2* genes are both overexpressed is an increase in the amount of prohibitin complex observed (Nijtmans *et al.*, 2000). These results show that overexpression of the prohibitin complex does not compensate for the absence of Tcm62p and restore SDH assembly and respiration competency.

Redundancy of protein function can often be detected genetically; when mutations are combined, the resulting phenotype is more severe than either single mutation alone. We therefore generated the triple deletion strain *Δphb1, Δphb2, Δtcm62*. As seen with the *Δtcm62* strain, the triple deletion strain does not grow on minimal glycerol medium and shows a slow growth phenotype on rich glycerol medium, with colonies only appearing after 3-4 days at 30 °C (Figure 3.2A). The observed phenotype is due to the *TCM62* deletion because respiratory competence can be restored by introduction of a plasmid-borne wild type *TCM62* gene. The better growth of the triple deletion strain compared to the *Δtcm62* strain can be attributed to differences in parental strain backgrounds. This is evident by the fact that the *Δtcm62* strain complemented with a centromeric plasmid containing the *TCM62* gene (pTCM62-7) still grows more slowly than the *Δphb1, Δphb2* strain. Thus, the growth phenotypes of the *Δtcm62* strain and of the *phb1, Δphb2, Δtcm62* strain are essentially indistinguishable (Figure 3.2A). We quantified the residual respiratory abilities of the mutant strains by growing them on YP media containing 0 to 0.5% galactose. On this medium, yeast has an initial fermentative phase followed by a respiratory phase. Mutants that are completely respiration deficient attain approximately 10% the growth yield of a wild type strain (Oyedotun and Lemire, 1997). The growth yields, monitored as the optical densities at 600 nm of late stationary phase cultures, are presented in Figure 3.2B. The triple deletion strain containing the empty vector pRS414 has a significantly reduced growth yield of ~38%, consistent with a partial respiratory competence and its slow growth on rich glycerol medium. The growth yields of the *Δphb1, Δphb2* and of the triple deletion strain, transformed with pTCM62-7 are similar. This confirms that the respiration deficiency in the triple deletion strain is a result of the

TCM62 deletion. The growth yield of the Δ*tcm62* strain is not significantly different from that of triple deletion strain, indicating that the *PHB* gene mutations do not exacerbate the respiratory deficiency caused by the *TCM62* deletion.

Although Tcm62p and the prohibitin complex share a number of similar properties, we did not detect any evidence of overlapping functions. Overexpression or loss of the *PHB* genes in a Δ*tcm62* background do not restore or aggravate, respectively, the respiratory deficiency induced by the absence of Tcm62p. Our results suggest that these two chaperones do not have common substrates; although the prohibitin complex participates in the assembly of MRC enzymes, it does not function in SDH assembly. These results indicate that Tcm62p and the prohibitin complex function as regulators of apoptosis through different mechanisms or pathways. By acting on multiple components of the MRC, this method of regulation may provide efficient modulation of MRC function during the apoptotic cascade. The MRC plays a vital role in the execution of apoptosis through the release of ROS and decreased ATP generation (Newmeyer and Ferguson-Miller, 2003). These chaperones may affect the assembly, structure and ultimately the function of the MRC enzymes and their ability to carry out their prescribed functions during the execution of apoptosis. An involvement of Tcm62p in apoptosis was first established when Bcl-x_L, an anti-apoptotic protein, was shown to suppress the Δ*tcm62* phenotype (Vander Heiden *et al.*, 2002). Subsequent experiments revealed that Tcm62p was able to inhibit mammalian cell death following growth factor withdrawal (Vander Heiden *et al.*, 2002). These findings implicate mitochondrial chaperones as another group of proteins involved in the apoptotic process, particularly ones associated with SDH. An increasing body of evidence suggests that SDH function is intimately involved both in apoptosis and tumorigenesis with a sensitivity to these processes often being inversely correlated (Albayrak *et al.*, 2003; Eng *et al.*, 2003; Ishii *et al.*, 2005). Tcm62p could therefore act as a regulator of apoptosis by modulating the levels of SDH holoenzyme or affecting the stability of the enzyme. Additional investigation will be required to understand how mitochondrial chaperones can act as regulators of apoptosis.

Another area that needs to be address is the mechanism of how Bcl-x_L complements the Δ*tcm62* mutant. The obvious area of investigation is to focus on whether Bcl-x_L expression restores SDH assembly and function. Bcl-x_L, based on its localization and previously characterized functions, is not expected to act as a chaperone and may promote SDH assembly indirectly. Improved coupling of metabolites between the cytosol and mitochondria as a result of Bcl-x_L expression may stimulate increased expression of SDH subunits and this overexpression may allow the enzyme to assemble in the absence of its chaperone. It would be assumed that this non-chaperone mediated assembly of SDH would be inefficient. This is in agreement with the observation that Bcl-x_L expression, although improving significantly the growth of the Δ*tcm62* mutant in glycerol-containing media, was not able to completely rescue this phenotype (Vander Heiden *et al.*, 2002).

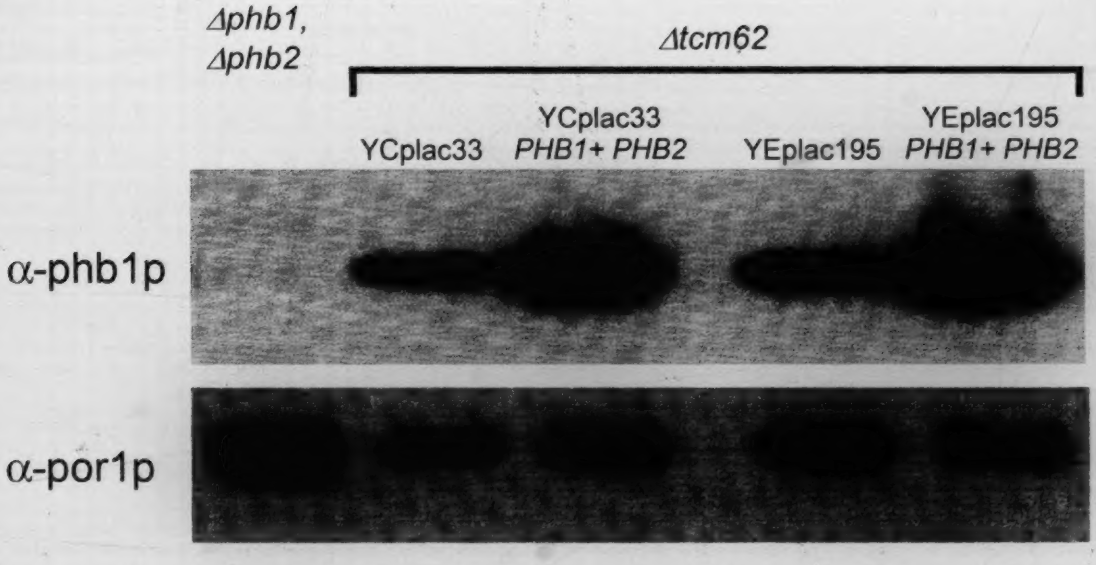
Δphb1,
Δphb2
Δtcm62
YCplac33
YCplac33
PHB1+ PHB2
YEplac195
YEplac195
PHB1+ PHB2
α-phb1p
α-por1p

Figure 3.1 Overexpression of prohibitin proteins in a *Δtcm62* background. Whole cell lysates were isolated from the *Δphb1, Δphb2* strain or the *Δtcm62* strain transformed with either the multi-copy vector, YEplac195, or the single copy vector, YCplac33, with or without the *PHB1* and *PHB2* genes. Whole cell lysates were subjected to Western blot analysis using antisera directed against the yeast Phb1p and Por1p.

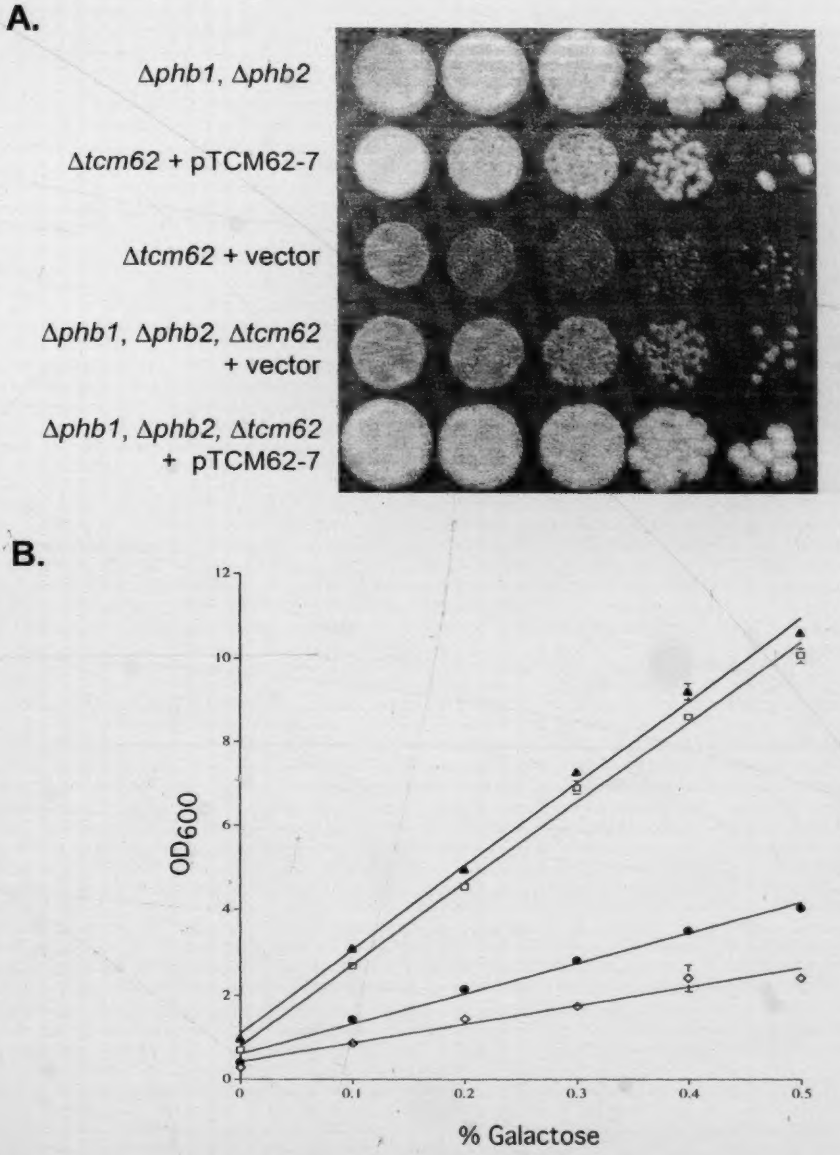

Figure 3.2 The respiration dependent growth of mutant *S. cerevisiae* strains. *A*, The strains *Δtcm62* and *Δphb1*, *Δphb2*, *Δtcm62* transformed a plasmid containing *TCM62* (pTCM62-7) or the empty vector (pRS414) and *Δphb1*, *Δphb2* were grown on SD medium at 30 °C, adjusted to the same cell densities, and 10-fold serial dilutions were plated onto YPG plates, which were incubated for 6 days at 30 °C. *B*, Yeast precultures were grown on SD medium at 30 °C for inoculation of YP media containing 0 - 0.5% galactose to a starting OD_{600} of 0.050. Triplicate cultures were grown to late stationary phase (~100 hours) and optical densities at 600 nm were measured. The yeast strains are: filled triangles, *Δphb1*, *Δphb2*, *Δtcm62* pTCM62-7; filled circles, *Δphb1*, *Δphb2*, *Δtcm62* pRS414; squares, *Δphb1*, *Δphb2*; diamonds, *Δtcm62* pRS414.

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CHAPTER 4

Ubiquinone-binding Site Mutations in the *Saccharomyces cerevisiae* Succinate Dehydrogenase Generate Superoxide and Lead to the Accumulation of Succinate

A version of this chapter has been published. Szeto, S. S. W., S. N. Reinke, B. D. Sykes and B. D. Lemire (2007) *J. Biol. Chem.* **282**: 27518-27526.

4.1 Introduction

Succinate dehydrogenase (SDH) is an essential enzyme linking the mitochondrial respiratory chain (MRC) and the tricarboxylic acid cycle. Since SDH participates in these two fundamental metabolic pathways, deleterious mutations that reduce enzyme function can produce severe physiological consequences. There are two classes of disorders associated with SDH deficiencies and these diseases are linked to a particular subset of SDH genes. Leigh syndrome, an infantile-onset progressive neurodegenerative disease, is caused by mutations in the human *SDHA* gene (Bourgeron *et al.*, 1995; Ackrell, 2002). While Leigh syndrome is the most common presentation, late onset optic atrophy, ataxia encephalopathy, and isolated cardiomyopathy have also been reported (Taylor *et al.*, 1996; Birch-Machin *et al.*, 2000; Davili *et al.*, 2007). Since this enzyme is a critical component of the cell's energy production machinery, it is not surprising that SDH deficiencies lead to these disorders. Interestingly, mutations in the human *SDHB*, *SDHC* and *SDHD* genes do not generate a similar phenotype. Instead they cause the formation of benign and malignant tumors. These include inheritable forms of cancer such as pheochromocytomas (catechol-secreting tumors commonly occurring in the adrenal medulla), paragangliomas (vascularized tumors in the head and neck) and renal cell carcinoma (Baysal *et al.*, 2002; Rustin and Rötig, 2002; Neumann *et al.*, 2004). More recently, it was reported that loss of the *SDHD* gene may contribute to gastric and colon cancers (Habano *et al.*, 2003). These observations highlight the importance of SDH genes as mitochondrial tumor suppressors (Gottlieb and Tomlinson, 2005). Moreover, the SDHC and SDHD subunits have also been implicated as regulators of apoptosis (Albayrak *et al.*, 2003; Ishii *et al.*, 2005).

Although SDH mutations have been implicated in various types of cancers, it is of particular interest that these mutations cause tumors of the carotid body. The main role of this organ is to act as a chemosensor of the oxygen concentration supplied by cerebral arteries to the brain and this implicates SDH as a potential oxygen sensor. Defects in this enzyme are thought to induce a pseudo-hypoxic effect, a condition in which cells activate a hypoxic response in the presence of oxygen. Cells in this condition undergo compensatory proliferation, resulting in the neoplastic transformation of this tissue. Although a link has been established, the molecular mechanism connecting SDH to the hypoxia signaling pathway is still unclear. It is also unknown whether the same oncogenic mechanism is occurring in other types of cancers associated with SDH defects.

There are currently three models that link SDH deficiencies and oncogenesis. The first model suggests that mitochondria-generated reactive oxygen species (ROS) contribute to nuclear DNA damage, mutagenesis and tumorigenesis. A transgenic mouse cell line expressing a mutant *SDHC* gene with a Val69Glu mutation overproduces ROS and can form benign tumors when injected into nude mice (Ishii *et al.*, 2005). The mutant SDH is subject to electron leakage to molecular oxygen and the formation of superoxide anions, which initiates oxidative damage to cellular macromolecules. SDH is not normally thought of as a major source of free radicals, but evidence of oxidative stress, shortened lifespan of model organisms, genomic instability and tumorigenesis associated with SDH mutations is accumulating (Ishii *et al.*, 1998; Guo and Lemire, 2003; Ishii *et al.*, 2005; Slane *et al.*, 2006; Walker *et al.*, 2006).

The second model linking SDH dysfunction and oncogenesis also involves mitochondrial ROS generation, but in this case, the ROS act as signaling molecules that lead to the stabilization of the transcription factor hypoxia-inducible factor 1-α (HIF-1α) (Guzy and Schumacker, 2006). Under normoxic conditions, the levels of HIF-1α are very low due to its constant degradation. HIF-1α degradation is signaled by its hydroxylation on two prolyl residues, a reaction catalyzed by prolyl hydroxylase (PH) enzymes (Figure 4.1). PH enzymes catalyze the conversion of proline residues, O_2 and α-ketoglutarate to hydroxy-proline, carbon dioxide and succinate using ascorbate and Fe^{2+} as cofactors. Under hypoxic conditions, PH activity is reduced and HIF-1α is stabilized. HIF activates a set of genes involved in angiogenesis, proliferation, cell survival and glycolysis (Pugh and Ratcliffe, 2003). High levels of ROS can induce a pseudo-hypoxic response by promoting the oxidation of Fe^{2+} to Fe^{3+}, limiting PH activity and HIF-1α modification and degradation (Gerald *et al.*, 2004).

In the third model, the signaling pathway involves succinate rather than ROS (Pollard *et al.*, 2005; Selak *et al.*, 2005; Selak *et al.*, 2006). Succinate accumulates as a result of SDH dysfunction and is believed to move out of the mitochondria via the dicarboxylate carrier. It induces a pseudo-hypoxic response and HIF-1α stabilization via product inhibition of the PH enzymes (Figure 4.1). Mutations in the fumarate hydratase gene *FH*, which encodes another enzyme of the tricarboxylic acid cycle, are also associated with tumorigenesis activated by a pseudo-hypoxic response; in the case of *FH* mutations, fumarate accumulates and inhibits PH activity (Isaacs *et al.*, 2005; Pollard *et al.*, 2005). High levels of succinate and the induction of HIF-1α have also been observed in *SDHA*-deficient fibroblasts although tumorigenesis has not been observed with *SDHA* mutations, probably because the expression of a second isoform of SDHA in paragangliomas precludes succinate buildup (Brière *et al.*, 2005).

In this study, we used the yeast SDH to gain further insight into the molecular mechanisms that link SDH function and tumorigenesis. We focused our attention on residues lining the Q_P site for two reasons. First, residues near the Q_P site are frequently mutated in paraganglioma and pheochromocytoma. In addition, the *Caenorhabditis elegans mev-1* mutant (a Gly71Glu substitution in SdhC) (Figure 4.2) shortens lifespan, promotes apoptosis and increases superoxide production (Ishii *et al.*, 1998). This mutation is also tumorigenic in a murine model and causes oxidative stress when modeled into the yeast SDH (Guo and Lemire, 2003; Ishii *et al.*, 2005). Second, we wished to further our understanding of the mechanism of ubiquinone reduction as our knowledge of this process in this enzyme is limited. A detailed understanding of quinone binding and reduction could potentially aid in the development of suitable treatments for human disease states.

The human *SDHC* R72C mutation affects a conserved arginine residue located in the Q_P site (Schiavi *et al.*, 2005; Bayley *et al.*, 2006). We mutated the equivalent residue Arg-47 in the yeast *SDH3* gene to Cys, Glu and Lys. Similarly, Asp-113 is a conserved amino acid in the human *SDHD* gene, also located in the Q_P binding site. Single amino acid substitutions of Asp-113 have not been reported in the SDH mutation database (Bayley *et al.*, 2005) but an Asp113 mutation in combination with a frameshift mutation is associated with tumor formation (Milunsky *et al.*, 2001; Neumann *et al.*, 2004). We mutated the equivalent yeast *SDH4* residue Asp-88 to Asn, Glu and Lys. The *SDH3* R47C and R47E mutations and the *SDH4* D88K mutation result in a loss of respiratory competence; the remaining mutations impair but do not abolish succinate-ubiquinone reductase activity. The *SDH3* R47K and the *SDH4* D88E and D88N mutations render the cells hypersensitive to oxygen and paraquat (methyl viologen). Using *in vivo* and *in vitro* assays, we determined that these mutations increase the production of superoxide anions. Thus, *SDH3* and *SDH4* mutations can be a significant source of oxidative stress. Finally, all of the mutants secrete succinate leading to highly elevated succinate to fumarate ratios in the medium. Our results confirm that the SDH Q_P site should be considered a significant source of ROS production and mutations that reduce SDH activity lead to elevated levels of succinate. These observations suggest that generation of both ROS and succinate may participate in delivering an oncogenic signal in human tumors.

4.2 Materials and Methods

Strains, media, culture conditions and isolation of submitochondrial particles - The *S. cerevisiae* strains sdh3W1 (MH125, *sdh3::TRP1*), sdh4W2 (MH125, *sdh4::TRP1*) and the *E. coli* strain DH5α have been described (Oyedotun and Lemire, 1997; Oyedotun and Lemire, 1999). sdh3W1/4K6 (YPH499, *sdh3::TRP1*, *sdh4::LYS2*) double knockout strain was constructed by targeted gene disruption (Rothstein, 1991). Briefly, a 0.13-kb KpnI fragment from the *SDH3* gene was replaced with a 0.83-kb KpnI fragment containing the *TRP1* gene in the yeast strain, sdh4K6 (YPH499, *sdh4::LYS2*) (Bullis and Lemire, 1994). The double knockout strain was verified by PCR and complementation analysis. The yeast media (YPD, YPG, SG, SD, and YPGal) have been described (Oyedotun and Lemire, 1997). Acid secretion was assessed using YPDBP medium containing 2.5 mM potassium phosphate pH 7.0 (de Kok *et al.*, 1975). All site-directed mutants were generated using a megaprimer mutagenesis method and confirmed by sequencing the entire gene as described (Sarkar and Sommer, 1990). Oxygen sensitivity was measured on plates incubated at 30 °C in a jar continuously flushed with pure oxygen at ~20 ml/min. Yeast precultures were grown on SD medium for 2 days to select for plasmid retention. YPGal medium was inoculated to a starting $OD_{600} = 0.1$ and grown at 30 °C for 3 days. Cells were harvested and lysed in a French pressure cell for the preparation of submitochondrial particles (Robinson *et al.*, 1991).

Free radical determination - Intracellular ROS production was examined using dihydroethidium (DHE) and MitoSOX Red (Molecular Probes). MitoSOX is a lipid-soluble cation that accumulates in the mitochondrial matrix where it can be oxidized to a fluorescent product by superoxide (Johnson-Cadwell *et al.*, 2007). DHE is uncharged and is not selectively accumulated in the mitochondrial matrix. As such, the fluorescent DHE oxidation product reports cytoplasmic and intermembrane space superoxide levels, although DHE may also be oxidized by hydrogen peroxide (Solans *et al.*, 2006; Johnson-Cadwell *et al.*, 2007). Both probes are cell-permeant and the red fluorescence of the oxidation products is enhanced by intercalation into DNA (Solans *et al.*, 2006).

Yeast strains were grown in SG medium containing 0.05% glucose to an optical density at 600 nm of ~2. Aliquots of 10^7 cells were washed twice with phosphate buffered saline (PBS) and incubated for 45 min at 25 °C in either 5 μM DHE or MitoSOX in the dark. Unlabeled cells were used to determine intrinsic fluorescence levels. Cells were washed three times and resuspended in PBS. Fluorescence was measured using a LS 50B luminescence spectrometer (Perkin Elmer, Boston, MA) with excitation and emission wavelengths of 510 and 580 nm, respectively.

Enzyme assays - SDH is normally isolated partially inhibited by oxaloacetate (Singer *et al.*, 1965). To activate the enzyme, submitochondrial particles were pre-incubated in 20 mM succinate for 15 min at 30 °C immediately prior to analysis. The malonate-sensitive succinate-dependent reduction of ubiquinone was monitored spectrophotometrically as the decylubiquinone (DB)-mediated reduction of dichlorophenol indophenol (DCPIP) (Kita *et al.*, 1989). The succinate-dependent, phenazine methosulfate (PMS)-mediated malonate-sensitive reduction of DCPIP is used as a measure of the membrane-associated catalytic dimer. Succinate and glycerol-phosphate-cytochrome *c* reductase assays were performed as described (Oyedotun and Lemire, 2001; Guo and Lemire, 2003).

Superoxide generation was assayed as the superoxide dismutase- (from bovine erythrocytes, catalog number 190117, MP Biochemicals) and malonate-sensitive reduction of cytochrome *c* in the presence of 1 mM potassium cyanide (Imlay and Fridovich, 1991). Ninety units of superoxide dismutase/ml were added to both sample and reference cuvettes. All assays were performed at room temperature.

^{1}H-NMR analysis of secreted acids - Yeast strains were grown overnight at 30 °C in YP medium containing 0.25% glucose. Cells were removed by centrifugation and the medium was subjected to trichloroacetic acid precipitation and centrifugation. The supernatant was recovered, adjusted to pH 7 with NaOH, flash frozen in liquid N_2 and stored at -80 °C until it could be lyophilized for 2 days and stored dry at 4 °C. The lyophilized material was dissolved in 570 μl D_2O (99.9%; Isotec Inc., Miamisburg, Ohio). Thirty μl of 5 mM 2,2-dimethyl-2-sila 3,3,4,4,5,5-hexadeuteropentane sulphonic acid (DSS-d_6) were added as a chemical shift reference and used as a concentration standard for determining absolute metabolite concentrations. pH was also recorded for calibration purposes. Samples were centrifuged at 14 000 x *g* for 3 min to remove particulate matter and 500 μl of the supernatant were transferred to a 5 mm NMR tube. All spectra were acquired on a 600-MHz Inova NMR spectrometer at 25 °C using the tnnoesy pulse sequence (circa Vnmr 6.1B software, Varian Inc). All spectra had an acquisition time of 4s, a preacquisition delay of 1s, a mixing time of 0.1s, a sweep width of 7200 Hz and 256 transients (Saude *et al.*, 2006). All spectra were analyzed using the Chenomx NMR Suite Professional software v4.5.

Miscellaneous methods - Measurements of FAD and protein contents, yeast and *E. coli* transformations and recombinant DNA methods have been described (Sambrook *et al.*, 1989; Bullis and Lemire, 1994).

4.3 Results

Choice of mutations – We chose to mutate residues in the yeast SDH Q_P site proposed to have important roles in ubiquinone binding or reduction (Figures 4.2 and 4.3) (Yankovskaya *et al.*, 2003; Oyedotun and Lemire, 2004). We mutated *SDH3* Arg-47 to Cys, Glu and Lys. The R72C mutation in the equivalent, conserved arginine of the human *SDHC* gene results in paraganglioma (Schiavi *et al.*, 2005; Bayley *et al.*, 2006). Mutation of Arg-31, the equivalent residue in the *E. coli* SdhC subunit results in respiratory deficiency and in the loss of succinate-quinone reductase activity (Yang *et al.*, 1998; Tran *et al.*, 2006).

We also mutated *SDH4* Asp-88 to Asn, Glu and Lys. Mutation of the equivalent *E. coli* SdhD Asp-82 abolishes succinate-quinone reductase activity and respiratory growth (Tran *et al.*, 2006). Both *SDH3* Arg-47 and *SDH4* Asp-88 may be functionally important for the protonation of ubiquinone upon reduction (Yankovskaya *et al.*, 2003; Sun *et al.*, 2005).

Growth phenotypes of the mutants - The mutant *SDH3* genes were cloned into the single copy shuttle vector YCplac33 and transformed into the *SDH3*-knockout strain sdh3W1. Similarly, mutant *SDH4* genes were cloned into the vector pRS416 and the resulting plasmids introduced into sdh4W2. To quantify each strain's ability to respire, growth yield assays on semisynthetic medium containing 0.1-0.5% galactose as the sole carbon source were performed (Figure 4.4). *S. cerevisiae* exhibits a diauxic shift when cultured on galactose; an initial fermentative growth is followed by a respiratory phase when the fermentable carbon source is limiting (Oyedotun and Lemire, 1999; Oyedotun and

Lemire, 2001; Oyedotun *et al.*, 2004). The growth yield of the sdh3W1 strain carrying a wild-type plasmid-borne *SDH3* gene, sdh3W1/pYCSDH3 was set at 100% (Figure 4.4A). The strain sdh3W1/pYCplac33 (empty vector) achieved by fermentation alone a growth yield of 12 ± 1%. The *SDH3* R47K mutant retained significant respiratory capacity with a 70 ± 6% growth yield. In contrast, the mutants R47C and R47E showed more severely impaired or abolished respiratory capacities with yields of 35 ± 4% and 12 ± 1%, respectively. We have previously noted that strains with a growth yield below approximately 30% are unable to grow on minimal glycerol media. Thus, the yeast R47C mutation and likely its counterpart human R72C mutation that results in paraganglioma are strong alleles that severely impair SDH activity.

The *SDH4* deletion strain containing the empty vector sdh4W2/pRS416 achieves a growth yield of 10 ± 1% of the wild-type (Figure 4.4B). A similar growth yield for the D88K mutant indicates a complete loss of respiratory growth. In contrast, the D88E and D88N mutants have yields of 80 ± 7% and 72 ± 9% respectively, demonstrating that they have retained significant respiratory capacity.

We examined the abilities of the mutant strains to grow when glycerol, a non-fermentable carbon source is available (Figure 4.5A and C, Fig. 4.6A and C). The *SDH3* R47C and R47E mutants were unable to grow while the R47K mutant grew as well as the wild-type. Consistent with the galactose growth yield assays, the *SDH4* D88K mutant is unable to grow on glycerol, while the D88E and D88N mutants displayed growth comparable to that of the wild-type.

The mutant enzymes assemble and have reduced quinone reductase activity – We used two measures to assess the effects of the mutations on enzyme assembly. First, we determined the membrane-associated succinate-dependent PMS-mediated reduction of DCPIP in isolated submitochondrial membranes as a measure of enzyme assembly. This activity is a measure of the membrane-associated catalytic dimer; it does not require catalytically competent Sdh3p and Sdh4p subunits, but the subunits must be present to mediate membrane association of the Sdh1p/Sdh2p catalytic dimer (Robinson *et al.*, 1991; Bullis and Lemire, 1994). We also determined the levels of covalent FAD; SDH is the major covalent flavoprotein in *S. cerevisiae* (Robinson and Lemire, 1995). All mutant enzymes are efficiently assembled (Table 4.1); the FAD contents of the mutants are similar to those of wild-type, with exception of the *SDH3* R47E and *SDH4* D88K mutants, which have somewhat reduced levels. The PMS-mediated DCPIP reductase activities of the mutants are also comparable to wild-type indicating the Sdh3p and Sdh4p subunits are efficiently anchoring the catalytic domain to the membrane. The *SDH3* and *SDH4* deletion strains display a residual level of activity (<5% of wild-type) consistent with the absence of assembled enzyme (data not shown). In contrast, the cytochrome *c* and the decylubiquinone reductase activities of the mutants are significantly reduced. Thus, the mutations impair catalytic activity involving quinone reduction rather than enzyme assembly. Even the mutations that do not abolish respiratory growth, such as the *SDH3* R47K, *SDH4* D88E and D88N mutations, show substantial losses of quinone reductase activity. These results are consistent with previously characterized Q_P site mutants (Oyedotun and Lemire, 1999; Oyedotun and Lemire, 1999; Oyedotun and Lemire, 2001; Guo and Lemire, 2003).

The mutant enzymes confer hypersensitivity to oxidative stress – Paraquat (methyl viologen) is an herbicide believed to induce oxidative stress through the generation of superoxide ions either in the cytoplasm (Yanase *et al.*, 2002) or in the mitochondria (Betarbet *et al.*, 2002). The *SDH3* R47K mutation renders the strain hypersensitive to 1.0 mM paraquat (Figure 4.5B), while the *SDH4* D88E and D88N mutations confer mild sensitivity to this agent (Figure 4.5D).

We also tested the effects of hyperoxia on respiratory growth. Wild-type *SDH3* or *SDH4* genes support slightly impaired growth in 100% oxygen as compared to normoxia (Figure 4.6). The *SDH3* R47K and *SDH4* D88N mutations abolish growth in the oxygen atmosphere; whereas the *SDH4* D88E mutation confers a milder sensitivity to hyperoxia (Figure 4.6D). We believe the mutant enzymes increase ROS production under normoxic conditions but that cellular defense mechanisms can detoxify the ROS. However, the imposition of an additional exogenous stress such as paraquat or hyperoxia overwhelms these defense mechanisms.

To more directly test this hypothesis, *in vivo* ROS generation was assessed with the oxidation-sensitive probes DHE and MitoSOX. We observed significantly elevated levels of DHE and MitoSOX fluorescence in all three mutants compared to their corresponding wild type strains (Figure 4.7). The *SDH3* R47K mutant displayed a 1.7-fold increase in DHE fluorescence and a 2.2-fold increase in MitoSOX fluorescence. The *SDH4* D88E mutant displayed 1.4- and 1.5-fold increases, while the D88N mutant displayed 1.7- and 2.3-fold increases, respectively. As a positive control, DHE and MitoSOX fluorescence were examined in wild-type cells grown in the presence of 0.5 mM paraquat. These cells had significantly increased DHE fluorescence compared to untreated cells but interestingly did not exhibit significant changes in MitoSOX fluorescence (Figure 4.7). These observations suggest that paraquat primarily generates cytosolic ROS in yeast cells.

The mutant enzymes are a source of superoxide radicals – The *SDH3* R47K and *SDH4* D88E and D88N mutants are sensitive to oxidative stress and display higher rates of ROS production *in vivo*. We assayed *in vitro* superoxide production as the superoxide dismutase-sensitive reduction of cytochrome *c* (Imlay and Fridovich, 1991; Guo and Lemire, 2003). There are two pathways of electron transfer to cytochrome *c*; in the first, electrons flow through SDH, ubiquinone and the ubiquinol-cytochrome *c* oxidoreductase (complex III) to cytochrome *c*; the second pathway involves electron leakage and the formation of superoxide anions, which can directly reduce cytochrome *c*. Superoxide dismutase will inhibit the second pathway by converting superoxide into hydrogen peroxide. As seen in Table 4.2, superoxide dismutase inhibits the rate of cytochrome *c* reduction demonstrating that a fraction of the cytochrome *c* is directly reduced by superoxide. The addition of more superoxide dismutase to the assays did not further decrease activity (data not shown). In wild-type mitochondrial membranes, the superoxide dismutase-sensitive portion of the cytochrome *c* reductase activity is small (< 3.5% of the total cytochrome *c* reductase activity) and not statistically significant. In contrast, that superoxide mediated portion of the cytochrome *c* reductase activity is greater for the mutant enzymes and is statistically significant. Although the mutant enzymes are much less efficient catalysts, they divert a larger fraction of their electron flow to the production of superoxide.

Complex III is a recognized source of ROS (Sun and Trumpower, 2003) and it has been shown that SDH and complex III have close structural and genetic interactions in yeast (Bruel *et al.*, 1996; Brasseur *et al.*, 1997). To determine whether the SDH mutations we created affected complex III-mediated ROS generation, we measured the glycerol-phosphate-dependent reduction of cytochrome *c* in the presence and absence of superoxide dismutase. Glycerol-phosphate is oxidized by the mitochondrial glycerol-phosphate dehydrogenase, an FAD-linked enzyme that, like SDH, reduces ubiquinone to ubiquinol (Bakker *et al.*, 2001). After the formation of ubiquinol, the pathway of electron transfer to cytochrome *c* is identical to that for the succinate-dependent reaction. If complex III function has been perturbed by the SDH mutations, we would also expect a large increase in superoxide production associated with glycerol-phosphate-cytochrome *c* reductase activity. As shown in Table 4.3, the addition of superoxide dismutase has no significant effect on this activity in wild-type or in mutant mitochondrial membranes. We conclude that SDH is directly responsible for the production of superoxide.

Enzymes possessing double mutations are catalytically inactive – We examined the effects of combining the *SDH3* and *SDH4* mutations in the same strain. Both of the double mutants we constructed (R47K/D88E and R47K/D88N) were unable to support respiratory growth (Figure 4.8A). The PMS-mediated DCPIP reductase activities of the double mutants indicate that the double mutant enzymes can assemble, although less efficiently than the wild-type, and are catalytically defective (Figure 4.8B).

SDH mutations lead to the accumulation and secretion of succinate – Inhibition of enzymes of the tricarboxylic acid cycle leads to the secretion of acid, which can be detected using pH-sensitive indicator dyes (de Kok *et al.*, 1975). Human SDH mutations that result in tumor formation have been shown to result in the accumulation of succinate (Pollard *et al.*, 2005; Selak *et al.*, 2005). Our data suggest that all of the mutations we created reduce or eliminate SDH activity, possibly leading to the accumulation and secretion of succinate. We qualitatively examined acid secretion using YPDBP plates, which contain bromocresol purple (Figure 4.9). Wild-type and mutant strains were spotted onto YPDBP plates along with the *Δtcm62* strain, a previously characterized acid secreting strain (Dibrov *et al.*, 1998). All mutant strains secreted acid as indicated by the halo of yellow colour surrounding the colonies. In contrast, the area surrounding the wild-type colonies remained purple. These results suggest that SDH mutations cause the secretion of organic acids.

Metabolite concentrations were determined from the 1D ^{1}H-NMR spectra using the Chenomx software. Individual metabolites were identified by comparison with spectra of standards and then absolute concentrations calculated by comparison to the added reference peak (Saude *et al.*, 2006). We identified succinate and fumarate in the ^{1}H-NMR spectra of the culture media after growth of the strains. Their concentrations were normalized to the optical densities of the respective cultures. As a control, we also analyzed the concentration of succinate and fumarate in the media prior to growth of the strains. We found that the concentration of succinate does not change substantially with the growth of the wild-type strains, consistent with the YPDBP plate assay (Figure 4.9). As seen in Table 4.4, the media used to grow the *SDH3* and *SDH4* deletion and mutant strains show significantly higher succinate to fumarate ratios when compared to media from their corresponding wild-type strains. These results confirm that SDH dysfunction can result in the accumulation and secretion of succinate.

4.4 Discussion

To gain further insight into the molecular basis of pathogenic SDH mutations, we investigated the role of Q_P site residues Sdh3p Arg-47 and Sdh4p Asp-88. Mutations around the Q_P site of an eukaryotic SDH can generate significant amounts of superoxide and can be tumorigenic (Ishii *et al.*, 1998; Guo and Lemire, 2003; Ishii *et al.*, 2005). Our results indicate that Sdh3p Arg-47 and Sdh4p Asp-88 are critical for quinone reduction. Substitution of these residues decreases quinone reductase activity (Table 4.1) and impairs respiratory growth (Figure 4.4). The loss of enzymatic activity in the mutants is not due to the disruption of SDH assembly as judged by covalent FAD contents and by membrane associated succinate-PMS/DCPIP activities (Table 4.1). The catalytic activities of the mutant enzymes were not stimulated by higher concentrations of DB, suggesting that the decrease in activity of these mutants can not be attributed to a lower affinity of the enzyme for ubiquinone (data not shown).

We observed an increase in superoxide production for the *SDH3* R47K, *SDH4* D88E and D88N mutants in comparison to the wild-type enzyme (Table 4.2). This superoxide generation was SDH-specific and was not the result of increased ROS production in complex III (Table 4.3). Our results are in agreement with previous data that suggest superoxide generation by SDH is not a flavin-mediated event but rather a quinone-mediated one (Guo and Lemire, 2003). It has been proposed that the FAD is the primary site of superoxide production in the *E. coli* SDH because excess succinate is able to suppress superoxide formation by occupying the catalytic site and obstructing access of oxygen to the reduced flavin (Messner and Imlay, 2002).

Several lines of evidence support the notion that superoxide generation by SDH is not a flavin-mediated event but rather a quinone-mediated one. Superoxide production was measured as a function of succinate concentration for the yeast SDH and more recently for the *E. coli* enzyme (Guo and Lemire, 2003; Zhao *et al.*, 2006). In both cases, the rates of superoxide production were highest at saturating succinate concentrations, suggesting that superoxide production is not at the active site. Addition of the competitive inhibitor pentachlorophenol (PCP), which binds to the *E. coli* SDH Q-site, significantly lowers the yield of superoxide. Binding of PCP is thought to reduce the availability of ubiquinone to mediate electron transfer to oxygen (Zhao *et al.*, 2006). In previous work, the affinities of the mutant enzymes for succinate were examined and found not to be significantly altered (Guo and Lemire, 2003). The similar apparent k_m values of the mutant and wild-type enzymes are not unexpected because the SDH active site is quite distant from the Q_P site where the mutations are located. This suggests that the enzyme's ability to bind succinate has not been perturbed, again arguing against FAD as the site of superoxide generation. These observations suggest that the Q_P site with bound ubiquinone is an important source of superoxide generation.

In vitro, the mutant enzymes produced superoxide at rates similar to the wild-type SDH; however, greater proportions of the total enzyme activity in the mutants were directed towards superoxide generation. We examined whether oxygen availability was limiting the rates of superoxide production. We repeated the cytochrome *c* reductase assays using oxygen saturated buffer but found no increase in superoxide production (data not shown). We suggest that the rates of superoxide production *in vivo*, where succinate concentrations are not saturating as they are *in vitro* (Mashego *et al.*, 2005), may be considerably higher for the mutant enzymes or alternatively, lower for the wild-type enzyme. The mutants are sensitive to oxidative stress and have higher rates of *in vivo* ROS production (Figures 4.5 – 4.7).

The SDH mutations we introduced may increase superoxide production in one of two ways. They may perturb the Q_P site allowing for increased electron flow out of the enzyme to oxygen, or they may extend the lifetime of the ubisemiquinone reaction intermediate, resulting in its premature release and subsequent reaction with oxygen to form superoxide. We cannot distinguish between these mechanisms, but favor the latter. When the corresponding *E. coli* SDH residues SdhC Arg-31 and SdhD Asp-82 were mutated, succinate-quinone reductase and plumbagin-fumarate reductase activities were both greatly affected, suggesting that these residues are critical to the quinone chemistry of the enzyme whether operating in forward or in reverse directions (Tran *et al.*, 2006).

Sdh3p Arg-47 in yeast and SdhC Arg-31 in *E. coli* are essential residues in their respective Q_P sites; in *E. coli*, Arg-31 has been proposed to modulate the pK_a of SdhD Y83, which is probably a proton donor during quinone reduction (Figure 4.3) (Yankovskaya *et al.*, 2003; Silkin *et al.*, 2007). The R47K mutation is predicted to raise the pK_a of the Tyr-89 hydroxyl group, reducing its propensity to donate a proton to the ubisemiquinone intermediate and increasing the lifetime of the intermediate. It was previously shown in the *E. coli* SDH that an arginine side chain is critical for proper quinone reduction and that substitution to lysine is inadequate (Yang *et al.*, 1998).

Sdh4p Asp-88 does not interact directly with bound quinone but is an essential component of the Q_P site. We propose that the D88E or the D88N mutations will perturb the electrostatic environment around Sdh3p Arg-47, resulting in the movement of Arg-47 away from Tyr-89. Asp-88 forms a hydrogen bond with Sdh2p His-217 and a salt bridge with Sdh3p Arg-47 (Oyedotun and Lemire, 2004; Huang *et al.*, 2005; Sun *et al.*, 2005). In *E. coli*, the equivalent histidine residue is believed to play an essential role in coordinating ubiquinone prior to electron transfer and protonation (Horsefield *et al.*, 2006). The D88N substitution removes the charge and the salt bridge with Arg-47. The D88E mutation increases the length of the side chain and our modeling studies suggest that the glutamic acid will move away from Sdh3p Arg-47 towards Sdh2p Arg-215 (data not shown). We propose that both of the D88E and D88N mutations will cause a reorientation of the Sdh3p Arg-47 side chain within the hydrophobic environment of the Q_P site because of the loss of the nearby stabilizing negative charge. Movement of the Arg-47 side chain will weaken its interaction with Sdh4p Tyr-89, raising that residue's pK_a and reducing its ability to function as a proton donor. The effect is similar to that of the Sdh3p R47K substitution. For the Sdh3p R47K and the Sdh4p D88E and D88N substitutions, ubiquinone binding is maintained, allowing electron transfer to proceed, but protonation of the ubisemiquinone by Tyr-89 is slowed. These effects may contribute not only to the lower quinone reductase activities observed in the mutants, but also their production of superoxide (Tables 4.1 and 4.2).

The SDH mutants we generated are acid secreting (Figure 4.9). Using ^{1}H-NMR, we showed that succinate is secreted and that the ratio of succinate to fumarate is much higher in media of the mutants than in media of wild-type strains (Table 4.4). These observations are consistent with the third model of SDH-linked tumorigenesis in which SDH dysfunction leads to elevated levels of succinate and HIF-1α stabilization. Oxidative stress may also contribute to the accumulation of succinate. In rat mitochondria treated with peroxides to induce oxidative stress, the nonenzymatic decarboxylation of α-ketoglutarate results in the formation of succinate (Fedotcheva *et al.*, 2006). A similar process may also be occurring in our mutants once the superoxide generated in the mitochondria is converted into hydrogen peroxide by the mitochondrial superoxide dismutase. The *SDH3*R47K and *SDH4* D88E mutants are most sensitive to oxidative stress and in agreement with this hypothesis, exhibit the highest succinate to fumarate ratios of all the samples examined.

During embryogenesis, most sympathetic neuronal precursor cells undergo apoptosis as growth factors become limiting (Lee *et al.*, 2005). This developmental apoptosis is mediated by the prolyl hydroxylase EglN3, whose activity is inhibited by succinate accumulation (Lee *et al.*, 2005). It has been proposed that SDH mutations cause pheochromocytoma because neuronal precursor cells carrying mutations escape developmental apoptosis (Lee *et al.*, 2005). HIF-1α activation will also confer a growth advantage to those surviving cells that go on to form tumors because they will be better adapted to the anaerobic state through enhanced vascularization and glycolytic capacity (King *et al.*, 2006).

Although succinate accumulation seems to be a major contributing factor to the development of these cancers, ROS production is also proposed to play a significant role. The generation of ROS results in oxidative damage to cellular components such as the DNA, leading to the gradual accumulation of mutations and eventual cellular transformation (Jackson and Loeb, 2001). Metabolic oxidative stress causes genome instability (Samper *et al.*, 2003; Slane *et al.*, 2006) and is an important factor in many aging-related diseases, including cancer (Finkel and Holbrook, 2000). Evidence that SDH is a major contributor to this mechanism is accumulating from various sources including this work (Ishii *et al.*, 1998; Guo and Lemire, 2003; Ishii *et al.*, 2005; Slane *et al.*, 2006; Walker *et al.*, 2006). ROS may have a less direct effect in comparison to succinate accumulation in regards to SDH-linked tumorigenesis but its contribution is potentially just as significant. ROS also participate in many other vital cellular functions including the induction and execution of apoptosis (Madeo *et al.*, 1999). However tumorigenesis is linked with inhibited, not enhanced cell death so how can a seemingly simple rise in ROS trigger such divergent responses? One explanation might be that these different biological outcomes reflect subtle differences in the level and duration of the oxidant burst or the cellular context that accompanies this oxidative stress (Finkel, 2003). Prolonged low levels of ROS production from defective enzymes such as SDH may cause the accumulation of damage resulting in cellular transformation but are insufficient for the induction of apoptosis.

In conclusion, we have provided evidence that ROS production and succinate accumulation both occur during SDH dysfunction and that both may contribute to tumorigenesis. Their relative importance to tumor formation and tumor cell proliferation may depend on the particular nature of the SDH mutation. Our data also strongly suggest that the SDH Q_P site is a likely source of superoxide.

Table 4.1

SDH assembly and activity in mitochondrial membranes

Strain	***Covalent FAD***[a]	***DCPIP reductase***[b]	***cytochrome c reductase***[c]	***decylubiquinone reductase***[d]
SDH3 WT	48 ± 4	4300 ± 200	2300 ± 100	4300 ± 400
SDH3 (R47C)	42 ± 2	4500 ± 500 (106%)[e]	370 ± 30 (16%)	310 ± 30 (7%)
SDH3 (R47E)	35 ± 1	3500 ± 600 (82%)	280 ± 30 (12%)	130 ± 50 (3%)
SDH3 (R47K)	44.3 ± 0.9	4900 ± 600 (115%)	780 ± 90 (34%)	740 ± 40 (17%)
SDH4 WT	41 ± 2	3800 ± 100	2300 ± 100	3700 ± 200
SDH4 (D88E)	42 ± 2	3600 ± 200 (95%)	1390 ± 80 (60%)	870 ± 80 (24%)
SDH4 (D88K)	20 ± 1	4400 ± 400 (116%)	280 ± 30 (12 %)	400 ± 100 (10%)
SDH4 (D88N)	39 ± 1	4700 ± 100 (124%)	890 ± 40 (39%)	600 ± 40 (16%)

[a] Values represent the mean of 5 trials ± S.D. FAD contents are expressed as pmol of FAD mg of protein $^{-1}$.

[b] Turnover numbers are expressed as μmol of PMS-mediated DCPIP reduced min^{-1} μmol of covalent FAD^{-1}. Values represent the mean of at least 10 trials ± S.D.

[c] Activities are expressed as μmol of cytochrome *c* reduced min^{-1} μmol of covalent FAD^{-1}.Values represent the mean of at least 12 trials ± S.D.

[d] Activities are expressed as μmol of decylubiquinone-mediated DCPIP reduced min^{-1} μmol of covalent FAD^{-1}. Values represent the mean of at least 10 trials ± S.D.

[e] Values in parentheses represent the percentage activities of the mutant enzymes compared to their respective wild-type controls.

Table 4.2

Superoxide dismutase-sensitive succinate-cytochrome *c* reductase activities

Strain	***SDH3 WT***	***SDH3(R47K)***	***SDH4 WT***	***SDH4(D88E)***	***SDH4(D88N)***
cytochrome *c* reductase[a] (- SOD)	108 ± 6	23 ± 2	86 ± 8	40 ± 3	31 ± 3
cytochrome *c* reductase[a] (+ SOD)	106 ± 6	21 ± 1	83 ± 6	37 ± 3	27 ± 2
SOD-dependent change	-1.9 %	-8.7 %	-3.5 %	-7.5 %	-12.9 %
P value[b]	0.49	≤ 0.001	0.34	≤ 0.01	≤ 0.001

[a] Activities are expressed as nmol of cytochrome *c* reduced min^{-1} mg of protein^{-1}. Values represent the mean of at least 12 trials ± S.D.

[b] Statistical significance was calculated using a 2-tailed unpaired Student's t-test.

Table 4.3

Superoxide dismutase-sensitive glycerol-phosphate-cytochrome *c* reductase activities

Strain	***SDH3 WT***	***SDH3(R47K)***	***SDH4 WT***	***SDH4(D88E)***	***SDH4(D88N)***
cytochrome *c* reductase[a] (- SOD)	60 ± 7	34 ± 1	50 ± 5	42 ± 2	56 ± 2
cytochrome *c* reductase[a] (+ SOD)	57± 7	32 ± 2	48 ± 5	40 ± 3	54 ± 3
SOD-dependent change	-5.0 %	-5.9 %	-4.0 %	-4.8 %	-3.6 %
P value[b]	0.23	0.03	0.37	0.13	0.02

[a] Activities are expressed as nmol of cytochrome *c* reduced min^{-1} mg of protein^{-1}. Values represent the mean of at least 12 trials ± S.D.

[b] Statistical significance was calculated using a 2-tailed unpaired Student's t-test.

Table 4.4

SDH mutants produce elevated amounts of succinate

Strain	[Succinate[a]]	[Fumarate[a]]	S : F Ratio
SDH3 WT	490 ± 100	7.7 ± 1.6	70 ± 20
SDH3 (R47C)	1200 ± 150	7.3 ± 0.9	160 ± 10***[b]
SDH3(R47E)	1340 ± 90	8.4 ± 1.3	160 ± 20***
SDH3 (R47K)	1200 ± 170	5.4 ± 0.8	230 ± 30***
SDH3 KO	1200 ± 110	8.0 ± 1.3	160 ± 30***
SDH4 WT	460 ± 180	8.6 ± 4.5	60 ± 30
SDH4 (D88E)	1020 ± 80	7.7 ± 3.9	160 ± 60*
SDH4 (D88K)	1300 ± 330	9.9 ± 3.5	140 ± 30**
SDH4 (D88N)	920 ± 100	4.3 ± 0.9	230 ± 60**
SDH4 KO	1100 ± 380	6.3 ± 2.3	180 ± 30***

[a] Values are expressed in μmol OD_{600}^{-1} and represent the mean of 5 trials ± S.D. Data was collected by Stacey N. Reinke.

[b] *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$ compared to the wild-type ratio using a 2-tailed unpaired Student's t-test.

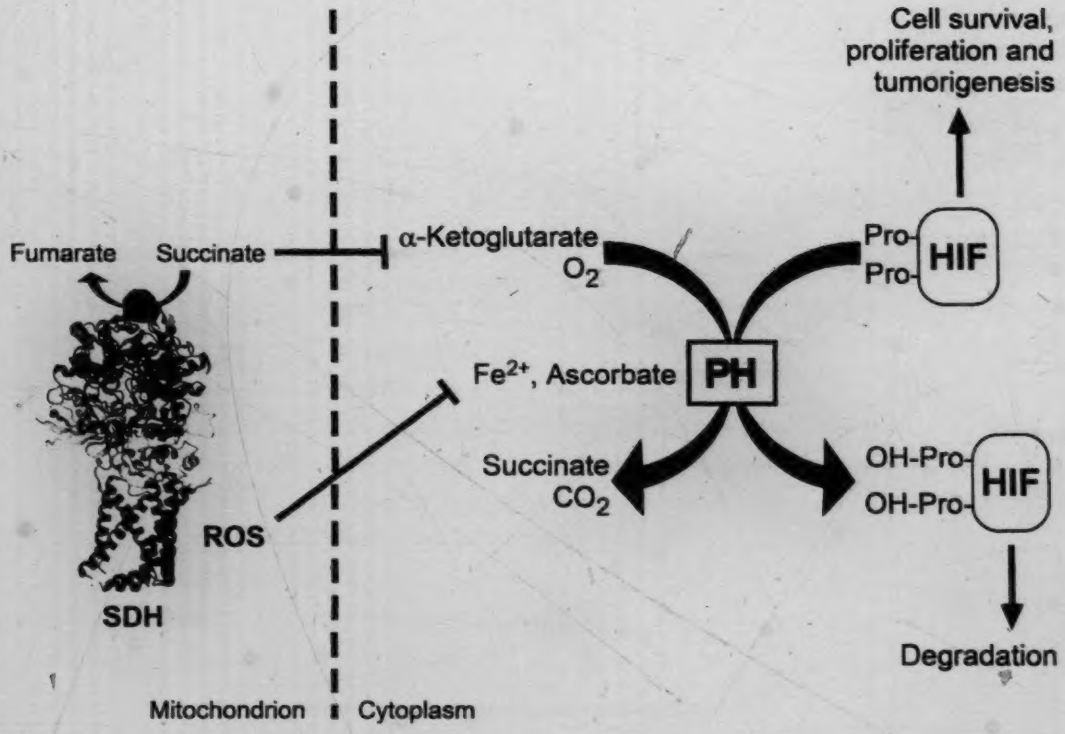
Cell survival, proliferation and tumorigenesis
Fumarate
Succinate
α-Ketoglutarate
O2
Pro-
Pro-
HIF
Fe2+, Ascorbate
PH
Succinate
CO2
OH-Pro-
OH-Pro-
HIF
ROS
SDH
Degradation
Mitochondrion
Cytoplasm

Figure 4.1 The mechanisms linking SDH dysfunction to oncogenesis via hypoxia-inducible factor induction. Hypoxia-inducible factor (HIF) 1-α degradation is signaled by hydroxylation on two prolyl residues. This reaction is mediated by prolyl hydroxylase (PH). PH catalyzes the conversion of proline residues, O_2 and α-ketoglutarate to hydroxy-proline, carbon dioxide and succinate using ascorbate and Fe^{2+} as cofactors. Reduced catalytic activity from mutant SDH causes the accumulation of succinate, which competes with α-ketoglutarate for binding to the active site of the enzyme resulting in PH inhibition. SDH dysfunction also leads to the generation of reactive oxygen species (ROS) that oxidize the cofactors required for PH function, causing its inactivation. Therefore, HIF-1α is stabilized and promotes the activation of genes involved in cell survival and proliferation resulting in tumorigenesis.

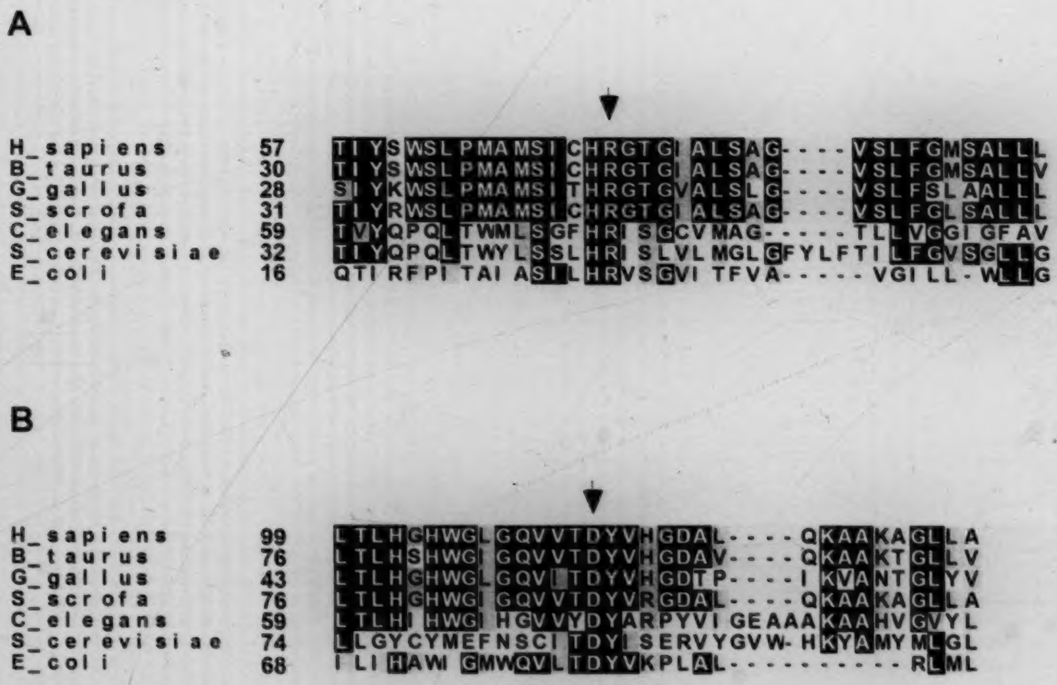
A
H_sapiens 57 TIYSWSLPMAMSICHRGTGIALSAG----VSLFGMSALLL
B_taurus 30 TIYSWSLPMAMSICHRGTGIALSAG----VSLFGMSALLV
G_gallus 28 SIYKWSLPMAMSITHRGTGVALSLG----VSLFSLAALLL
S_scrofa 31 TIYRWSLPMAMSICHRGTGIALSAG----VSLFGLSALLL
C_elegans 59 TVYQPQLTWMLSGFHRISGCVMAG-----TLLVGGIGFAV
S_cerevisiae 32 TIYQPQLTWYLSSLHRISLVLMGLGFYLFTILFGVSGLLG
E_coli 16 QTIRFPITAIASILHRVSGVITFVA-----VGILL-WLLG
B
H_sapiens 99 LTLHGHWGLGQVVTDYVHGDAL----QKAAKAGLLA
B_taurus 76 LTLHSHWGIGQVVTDYVHGDAV----QKAAKTGLLV
G_gallus 43 LTLHGHWGLGQVITDYVHGDTP----IKVANTGLYV
S_scrofa 76 LTLHGHWGIGQVVTDYVRGDAL----QKAAKAGLLA
C_elegans 59 LTLHIHWGIHGVVYDYARPYVIGEAAAKAAHVGVYL
S_cerevisiae 74 LLGYCYMEFNSCITDYISERVYGVW-HKYAMYMLGL
E_coli 68 ILIHAWIGMWQVLTDYVKPLAL----------RLML

Figure 4.2 Sequence alignments of Sdh3p and Sdh4p residues near the Q_P site.
A, Alignment of Sdh3p and SdhC subunits. *B*, Alignment of Sdh4p and SdhD subunits. Sequences are shown from *Homo sapiens*, *Bos taurus*, *Gallus gallus*, *Sus scrofa*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Escherichia coli*. Residues examined in this study are indicated with arrows. The residue number of the first amino acid in each aligned sequence is shown. Identical residues are highlighted in black and similar residues are highlighted in grey. The alignments were generated using Clustal X (Thompson *et al.*, 1997) and Boxshade 3.21.

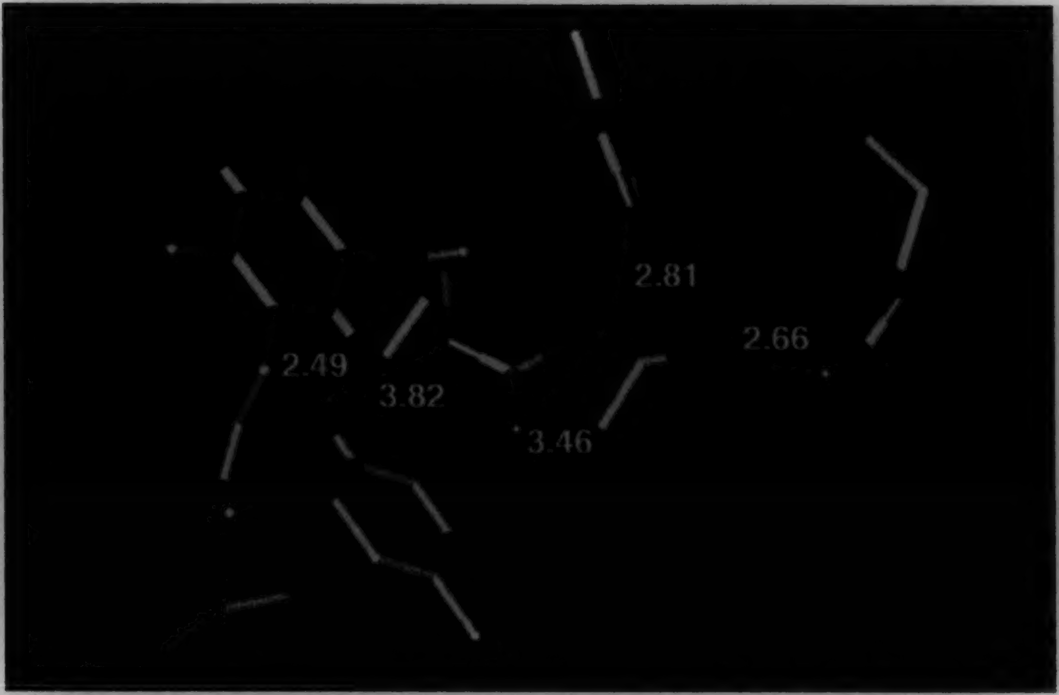

Figure 4.3 Location of the Sdh3p Arg-47 and Sdh4p Asp-88 residues at the Q_P site. Ubiquinone-2 was docked into a model of the yeast SDH (Oyedotun and Lemire, 2004). Only side chains are shown. Included are residues Sdh2p His-217 and Sdh4p Tyr-89, which interact with the residues examined in this study. Distances (shown in Å) between the side chains and the quinone are indicated by the *dashed yellow lines*.

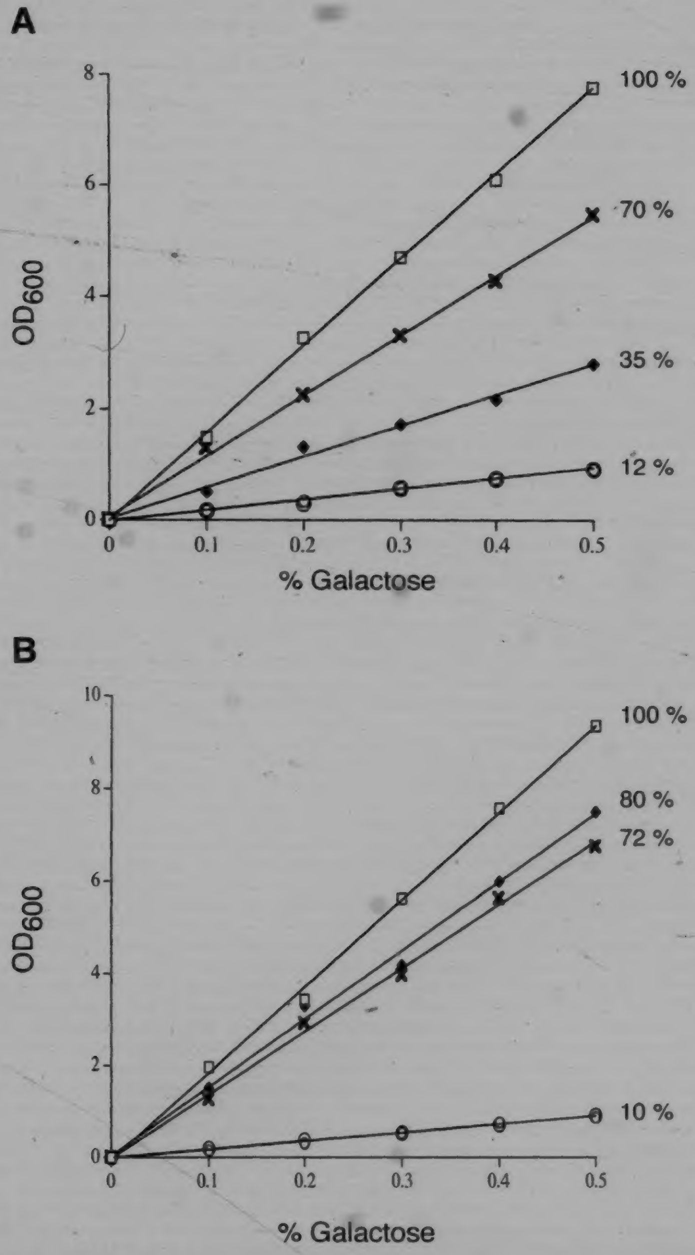

Figure 4.4 Growth of *S. cerevisiae* strains on galactose media. Yeast strains were grown at 30 °C on semisynthetic liquid medium containing 0.1-0.5% galactose. Cultures were inoculated at a starting OD_{600} of 0.05 and measured after reaching late stationary phase (approximately 100 h). The relative growth yields were calculated using the final absorbance values on 0.5% galactose. *A*, growth of *SDH3* mutant strains: *squares*, sdh3W1/pYCSDH3; *crosses*, R47K; *diamonds*, R47E; *circles*, R47C and sdh3W1/pYCplac33. *B*, growth of *SDH4* mutant strains: *squares*, sdh4W2/pSDH4-21; *diamonds*, D88E; *crosses*, D88N; *circles*, D88K and sdh4W2/pRS 416.

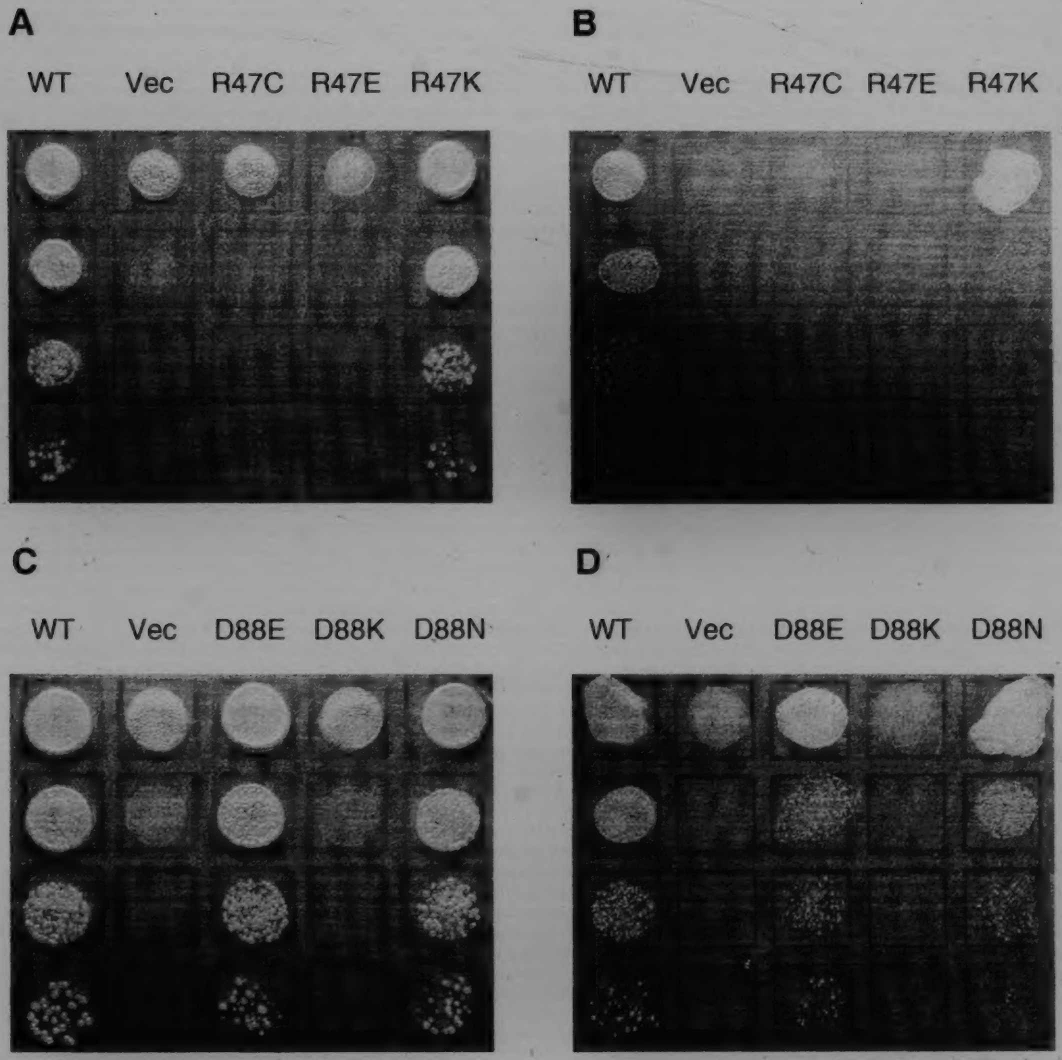
A
WT
Vec
R47C
R47E
R47K
B
WT
Vec
R47C
R47E
R47K
C
WT
Vec
D88E
D88K
D88N
D
WT
Vec
D88E
D88K
D88N

Figure 4.5 Respiratory growth of wild-type and mutant strains in the presence and absence of paraquat. *SDH3* (*A* and *B*) or *SDH4* (*C* and *D*) knockout strains transformed with wild-type (WT) or with empty vectors (Vec) or mutant *SDH3* or *SDH4* genes were grown overnight at 30 °C in SD. Cultures were serially diluted with sterile water and 5 µl aliquots were spotted onto SG plates without (*A* and *C*) or with (*B* and *D*) 1.0 mM paraquat. Plates were incubated at 30 °C and photographed after 6 days for control plates and after 12 days for paraquat plates.

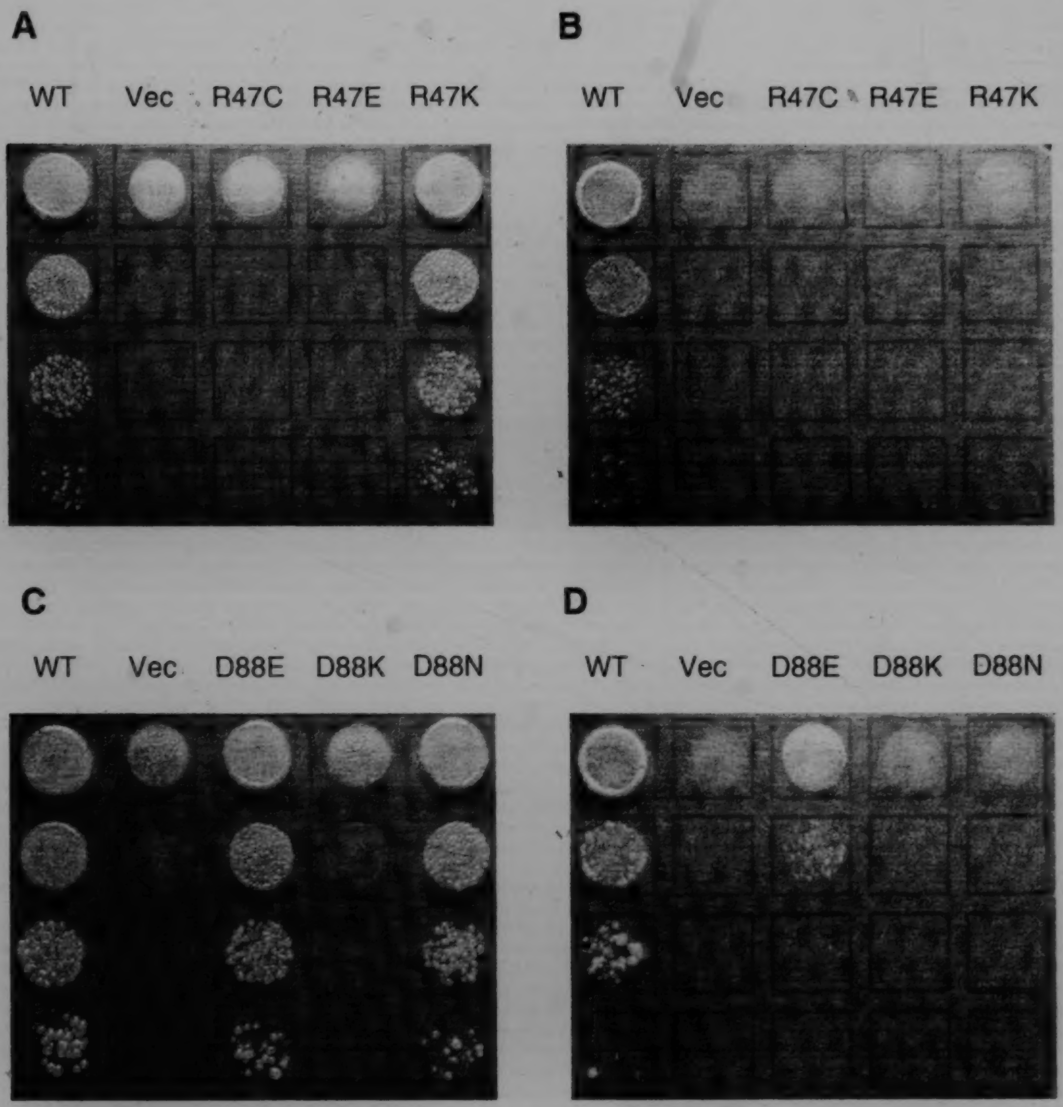
A
WT
Vec
R47C
R47E
R47K
B
WT
Vec
R47C
R47E
R47K
C
WT
Vec
D88E
D88K
D88N
D
WT
Vec
D88E
D88K
D88N

Figure 4.6 Respiratory growth of wild-type and mutant strains under normoxic or hyperoxic conditions. *SDH3* (*A* and *B*) or *SDH4* (*C* and *D*) knockout strains transformed with wild-type (WT) or empty vectors (Vec) or mutant *SDH3* or *SDH4* genes were grown overnight at 30 °C in SD. Cultures were serially diluted with sterile water and 10 µl aliquots were spotted onto SG plates. Plates were incubated at 30 °C in an air atmosphere and photographed after 6 days (*A* and *C*) or in a tank continuously flushed with 100% oxygen and photographed after 7 days for *SDH3* strains (*B*) and 6 days for *SDH4* strains (*D*).

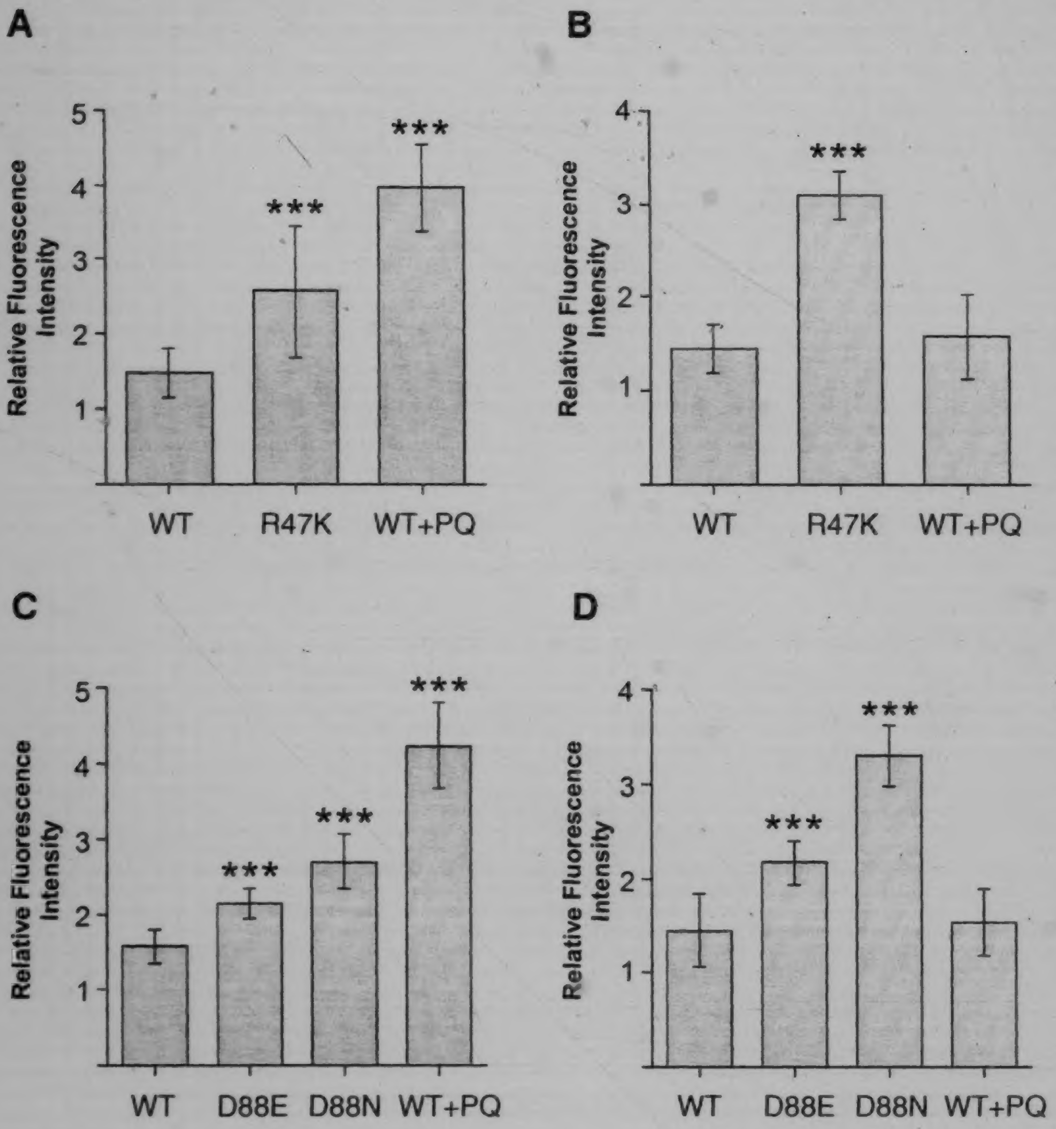

Figure 4.7 Detection of free radical production *in vivo* using fluorimetric probes. ROS production was determined fluorimetrically by examining the oxidation of DHE and MitoSOX as described under Materials and Methods. The relative fluorescence intensity was measured for *SDH3* (*A* and *B*) or *SDH4* (*C* and *D*) strains stained with either DHE (*A* and *C*) or MitoSOX (*B* and *D*). Values represent the mean ± S.D of eight trials. ***, P<0.001 compared to the wild-type value using a 2-tailed unpaired Student's t-test.

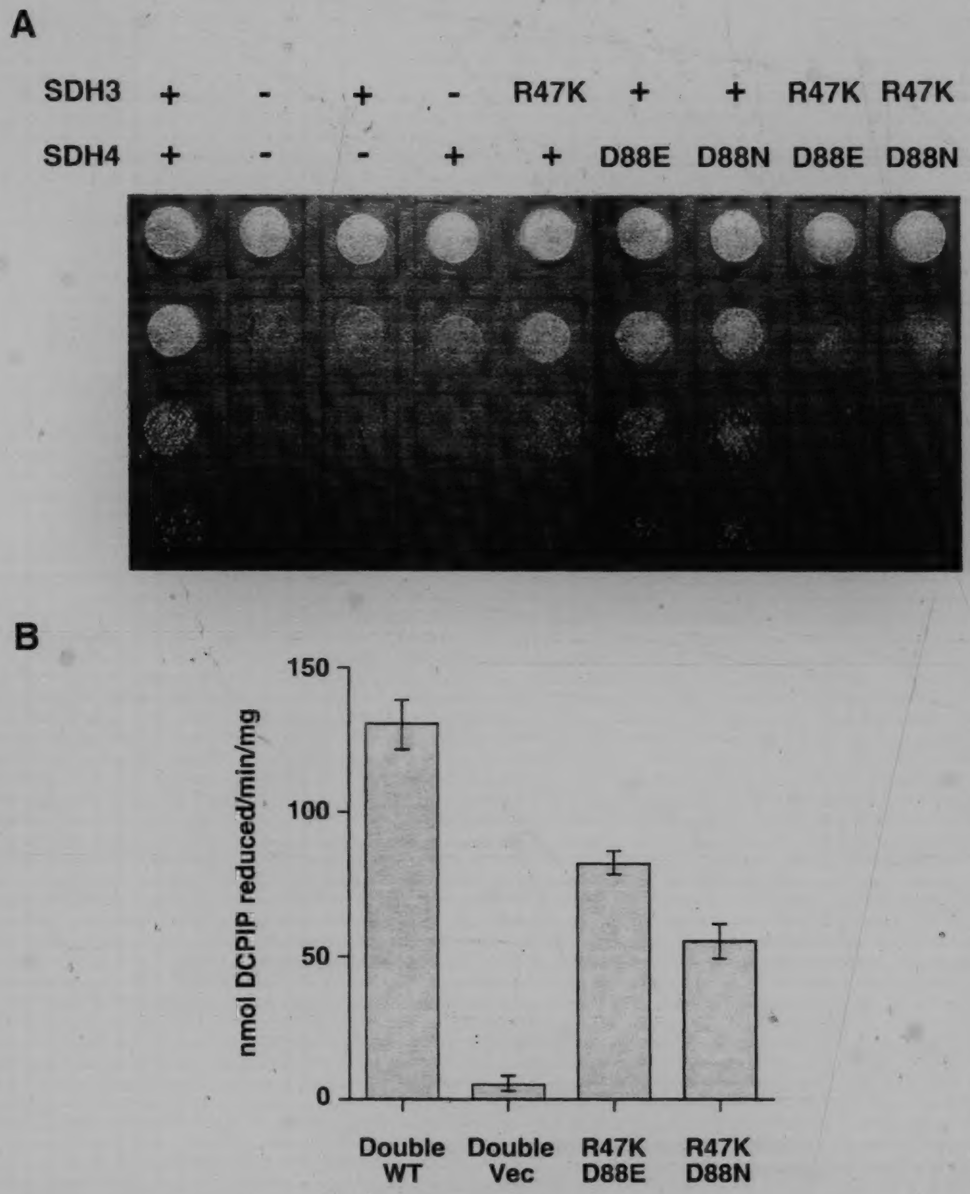

Figure 4.8 Double mutants contain assembled but catalytically inactive enzymes. *A*, the *SDH3* and *SDH4* double knockout strain transformed with wild-type (+), empty vectors (-) or mutant *SDH3* or *SDH4* plasmids were grown overnight at 30 °C in SD. Cultures were serially diluted with sterile water and 10 µl aliquots were spotted onto SG plates. Plates were incubated at 30 °C and photographed after 8 days. *B*, Succinate-dependent malonate-sensitive PMS-mediated DCPIP reductase activity was assayed on membranes from double wild-type, double empty vector or double mutant strains. Activities are expressed as nmol of PMS-mediated DCPIP reduced min^{-1} mg protein^{-1}. Values are means ± S.D of seven trials.

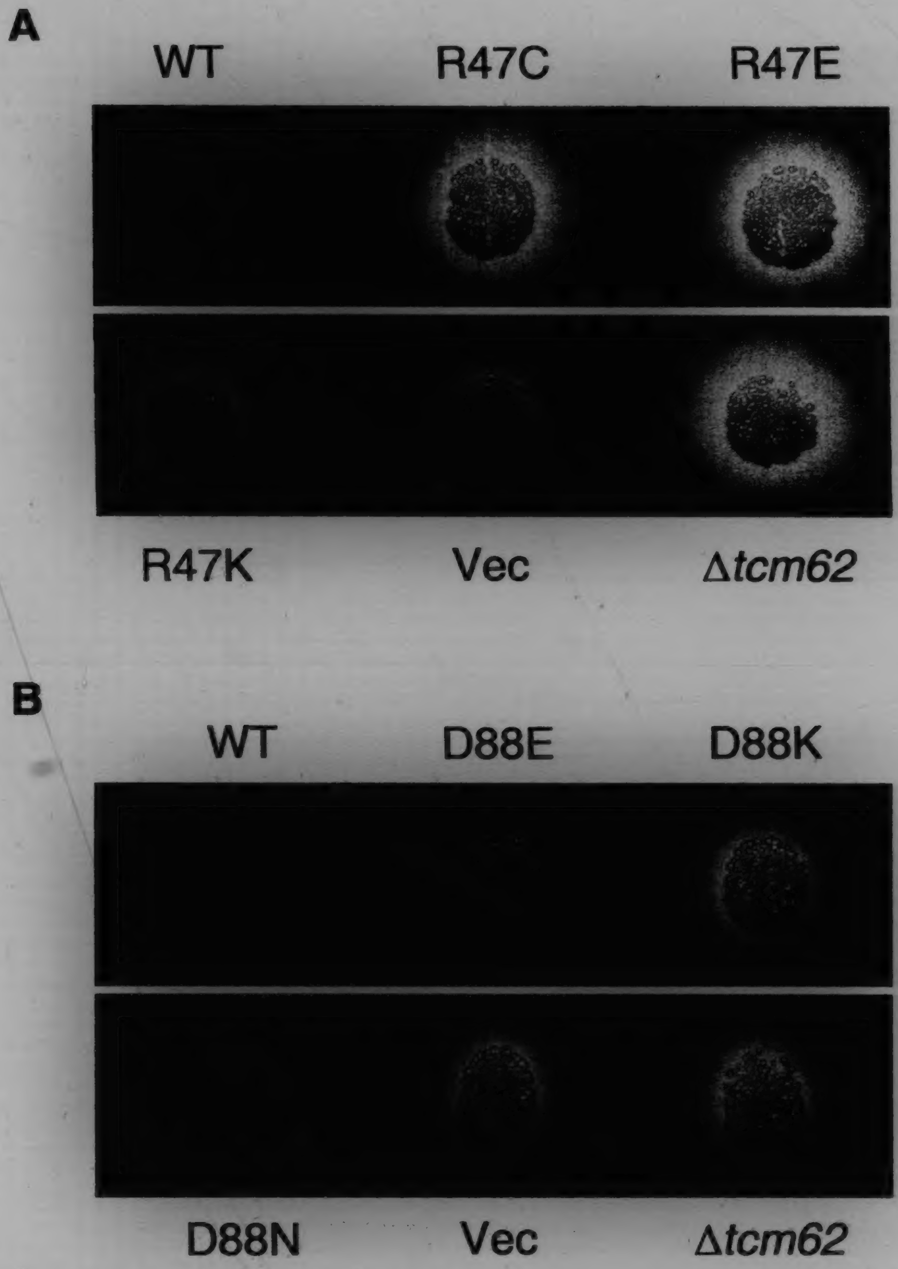
A
WT
R47C
R47E
R47K
Vec
Δ*tcm62*
B
WT
D88E
D88K
D88N
Vec
Δ*tcm62*

Figure 4.9 SDH mutants secrete acid. *SDH3* (*A*) or *SDH4* (*B*) knockout strains transformed with wild-type (WT) or with empty vectors (Vec) or mutant *SDH3* or *SDH4* genes were grown overnight at 30 °C in SD. The strain *Δtcm62* was used as a positive control (Dibrov *et al.*, 1998). Cultures were diluted 1,000 fold with sterile water and 10 µl aliquots were spotted onto YPDBP plates containing 2.5 mM potassium phosphate pH 7.0. Plates were incubated at 30 °C and photographed after overnight incubation.

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CHAPTER 5

Conclusions and Future Directions

5.1 Introduction

The aim of this thesis is to provide insight into the structure and function of SDH and its chaperone, Tcm62p. Studies involving the biogenesis and function of SDH are pertinent to human biology because of the enzyme's importance in energy metabolism and in the development of human disease. The high degree of structural and functional conservation amongst eukaryotic SDHs increases the likelihood that the yeast model system is biochemically relevant to human biology. Our work on Tcm62p has indicated that it is an integral membrane protein and that it exists as a component of several large multisubunit complexes. Examination of the structure and function of SDH has provided insight into the molecular mechanisms that link SDH function and tumorigenesis. In addition, we have improved our understanding of the mechanism of ubiquinone reduction, although it remains incomplete.

In this final chapter, I will discuss some ideas for future studies in the areas of SDH structure, function and assembly. The major areas of investigation I would draw attention to are the roles of chaperones in assembly, the role of heme in SDH structure and catalysis, the role of the Q_D site, the mechanisms of regulating SDH function and the participation of SDH in the essential cellular processes of higher eukaryotes related to energy production, apoptosis and tumorigenesis. Finally, I will discuss the advantages and disadvantages of the yeast model system for these future studies and propose the use of the *Caenorhabditis elegans* system for specific problems.

5.2 Discussion and Future Directions

5.2.1 Further Investigations Into the Structure and Function of Tcm62p and Its Role in SDH Assembly

Although some progress was made in the characterization of Tcm62p, much still remains to be discovered about the function of this protein and its role in SDH assembly. The results reported in Chapter 2 indicate that Tcm62p exists as a component of multisubunit complexes. Previous characterization of the protein has also ascribed various functions to it. Unfortunately, very little is known about the molecular details of these functions. We hypothesized that Tcm62p was an ATP-binding protein and mutated residues that might have a role in nucleotide binding. Our results suggest that Tcm62p does not require nucleotide binding for function. A goal of future studies should be to identify and characterize residues that are essential for Tcm62p structure and function by using random and site-directed mutagenesis approaches. Identifying essential residues will provide a greater understanding of the protein-protein interactions that exist between Tcm62p and other components of the high molecular weight complexes it is found in. In addition, some essential residues should affect chaperone function without impairing the protein's ability to oligomerize. Characterization of Tcm62p will lead to insight into the steps of SDH assembly that require chaperone involvement.

Currently, there is only one defined SDH-specific chaperone. The presence of high molecular weight complexes containing Tcm62p suggests that additional proteins may be involved in SDH assembly. Our initial attempts to identify these proteins using mass spectrometry were unsuccessful. Identifying additional chaperone proteins would provide significant insight into SDH biogenesis and would serve as a springboard for many additional studies. One approach for identifying additional chaperone proteins would be to screen the collection of yeast deletion mutants. In the *Saccharomyces* Genome Database, deletions of 516 loci inhibit growth on non-fermentable carbon sources, indicating a defect in respiration. Of these genes, 75 have not been characterized and have no known function. The genes for which we have information about are involved in various aspects of mitochondrial function, such as oxidative phosphorylation, the Krebs cycle, mitochondrial protein synthesis and transport, ionic homeostasis and the metabolism of vitamins, cofactors and prosthetic groups (Steinmetz *et al.*, 2002). The large majority of these genes do not participate in SDH biogenesis and have clearly defined functions. Chaperones may have overlapping functions and some proteins, like those involved in Fe-S cluster biosynthesis participate in the biogenesis of numerous enzymes (Lill and Kispal, 2000). Combining a plate assay for acid secretion with metabolite analysis as employed in Chapter 4, it should be possible to quickly screen candidate deletion mutants for defects in SDH assembly. Acid secretion is a hallmark of Krebs cycle dysfunction (de Kok *et al.*, 1975) and should eliminate the majority of respiratory deficient mutants not affecting the cycle. The metabolite analysis would allow for the detection of succinate accumulation, which is indicative of a defect in SDH biogenesis. Further characterization of mutants identified in this screen would be needed to define the specific steps in SDH biogenesis that are impaired.

An alternative approach for identifying proteins that interact with Tcm62p would be to utilize the collection of antibodies generated towards previously characterized mitochondrial proteins. The focus should be on inner membrane and matrix proteins as candidate targets based on Tcm62p's localization and topology. Using purified Tcm62p-containing complexes, we would screen all available antibodies and examine whether any candidate proteins have been co-purified. Reciprocal co-immunoprecipitation would verify the interaction of these proteins with Tcm62p. The advantage of this approach is that it would provide immediate insight into the mechanism of SDH assembly, since these proteins have been previously characterized.

Chaperone-mediated assembly of SDH is likely to occur not only in yeast but in other eukaryotic species as well. However, close homologs of Tcm62p have not been identified. To find functional homologs of Tcm62p, we will screen cDNA libraries from various organisms to search for genes that complement the *Δtcm62* mutant. This approach could also be applied to any other proteins that are found to interact with Tcm62p. Our ultimate goal is to identify the corresponding human genes for proteins involved in the assembly of SDH. This would provide additional candidate genes for sequencing of patients with SDH deficiencies without identified mutations.

5.2.2 Further Investigations Into the Structure and Function of SDH

Although significant progress has been made in the understanding of the structure and function of the SDH complex, several questions remain unanswered. Underlying these studies is the requirement for an efficient purification method for the yeast SDH. The availability of purified enzyme would facilitate more extensive biochemical and biophysical characterization of interesting mutants. SDH contains several essential redox active centers and the ability to examine them biophysically using techniques such as electron paramagnetic spectroscopy (EPR) would greatly enhance our understanding of electron transport and catalysis in the enzyme. The bovine heart SDH is an extensively characterized enzyme for which a wealth of biophysical information is available (Ackrell *et al.*, 1992). However, the bovine system is not amenable to genetic manipulation, limiting the information that can be acquired. This limitation is particularly evident in regards to disease-causing mutations that are thought to affect redox active centers (Ackrell, 2002). The *E. coli* enzyme has provided significant insight into the biophysical properties of SDH but this system lacks important features exhibited only in eukaryotes. Yeast is ideal for studying these disease-causing mutations as it is a eukaryotic system amenable to genetic manipulation. Previous attempts to overexpress SDH in yeast and in *E. coli* have proven unsuccessful. An alternative approach to overexpression is to purify native SDH through affinity chromatography. The C-termini of one or more SDH subunits could be epitope tagged with a hexahistidine tag or with a calmodulin-binding peptide tag to facilitate purification. These tags are chosen because of their short lengths and their utility under non-denaturing purification conditions (Klein, 2003). Tagging the Sdh2p subunit might be first considered, as its C-terminus protrudes into the aqueous medium (Oyedotun and Lemire, 2004; Sun *et al.*, 2005; Huang *et al.*, 2006). The tagged enzyme should be compared with the wild-type to ensure that epitope tagging does not affect the catalytic function of SDH. If a single tag approach is insufficient to isolate the enzyme to the required purity, then a combined, dual tag approach could be entertained.

The biophysical characterization of mutants with decreased stability can be a difficult undertaking. Solubilization and purification conditions tolerated by the wild type enzyme may be too harsh and cause the loss of cofactors or denaturation. This would be a likely scenario for heme-deficient mutants (Oyedotun *et al.*, 2007). Ideally, these mutants would be characterized while membrane bound. However, there can be significant interference from other membrane constituents, particularly when examining heme content. A much larger amount of heme in complex III masks the SDH-dependent heme signal in traditional dithionite-reduced minus ferricyanide-oxidized spectra (Hatefi and Galante, 1980). Spectroscopic examination of the heme *b* in the yeast SDH can be performed by adding fumarate to selectively oxidize the heme (Oyedotun and Lemire, 1999). This method works well when examining the wild-type SDH but poses a problem for mutants unable to reduce fumarate, leading to inaccurate heme quantitation. Such SDH mutants could be expressed in strains lacking mtDNA (*rho*°). These strains do not have an assembled MRC because genes encoding essential subunits of complexes III, IV and V are found in the mtDNA. SDH is encoded by four nuclear genes so its expression is not dependent on the mtDNA. Preliminary evidence indicates that the 3Fe-4S and 2Fe-2S clusters are detectable using EPR spectroscopy and that a signal corresponding to the heme in SDH is present in *rho*° cells (Guo, Rothery and Lemire, unpublished data). Further studies are required to determine whether this approach will be a useful method for characterizing the biophysical properties of SDH.

Clarifying the presence and role of a second quinone binding site is fundamental to fully understanding catalysis in SDH (Oyedotun and Lemire, 2001). The recent determination of the porcine and avian SDH crystal structures will no doubt further stimulate investigations into these questions (Sun *et al.*, 2005; Huang *et al.*, 2006). The sequences of the Sdh3p and Sdh4p homologs may also help to identify residues that are important for catalytic function. Preliminary evidence indicates that the Sdh3p and Sdh4p homologs are able to functionally replace their corresponding authentic subunits (Oyedotun and Lemire, unpublished data). By comparing the primary sequences of authentic and homolog subunits, it may be possible to identify functionally conserved residues and test their roles in catalysis by creating appropriate site-directed mutants. These mutants should be examined for changes in catalytic activity, changes in inhibitor sensitivity or binding, as well as changes in their sensitivity to oxidative stress using approaches described in Chapter 4. Any mutants with altered sensitivity to oxidative stress should be examined enzymatically for the production of ROS. ROS generation would raise the possibility that a semiquinone radical is formed at the Q_D site and would provide evidence that the site is electronically connected to other redox cofactors in the enzyme. Ultimately, the detection of a semiquinone radical by EPR spectroscopy would provide the strongest evidence for a functional Q_D site.

The role of the heme in SDH has long been enigmatic and several hypotheses have been proposed. These include electron transfer from succinate to the ubiquinone and/or a structural role in enzyme assembly or stability (Cecchini, 2003). Previous studies have explored these roles for the heme but several questions still remain. It has also been previously proposed that the heme acts as an electron sink to minimize free radical production (Cecchini, 2003; Yankovskaya *et al.*, 2003). This hypothesis is based on the observation that the *E. coli* fumarate reductase (FRD), which does not possess a heme, generates significant quantities of ROS when operating in the direction of succinate oxidation (Imlay, 1995). Examining the sensitivity of heme mutants, particularly a hemeless enzyme to oxidative stress might constitute a first step. Any mutants with altered sensitivity to oxidative stress should be examined enzymatically for the production of ROS.

Another role that has been ascribed to the heme is its involvement in fumarate reduction in the eukaryotic enzyme (Cecchini, 2003). The bovine SDH exhibited FRD activity when the mitochondrial inner membrane was in a highly energized state or when the purified enzyme was analyzed under anaerobic conditions (Yu *et al.*, 1987). The complex II in the adult nematode *Ascaris suum* also possesses FRD activity and functions as a key enzyme in anaerobic respiration (Kita *et al.*, 2003). The reverse function of complex II is also a physiologically relevant process as it is important in the recovery of tissues from ischemia-reperfusion and the initiation of hypoxia-induced vasoconstriction in the pulmonary vasculature (Weinberg *et al.*, 2000; Paddenberg *et al.*, 2003; Paddenberg *et al.*, 2003). Fumarate reductase activity of SDH has been characterized directly for both the *E. coli* and bovine enzymes (Kotlyar *et al.*, 1992; Grivennikova *et al.*, 1993; Maklashina and Cecchini, 1999). The ability of heme mutants to catalyze fumarate reduction should be investigated to determine whether heme participates in this process. The redox potential of the heme determines its ability to participate in catalysis. This may also be true for when the enzyme operates in the reverse mode. It will be important to determine the midpoint potential of the heme in the yeast SDH and to examine mutants that alter that potential. Changing the electrostatic environment around the heme and its midpoint potential could be achieved by mutating residues in close proximity to the heme. Effects on enzyme function in both the forward and reverse directions would be examined.

In Chapter 4, we used the yeast SDH as a model system for investigating the molecular mechanisms of SDH-associated tumorigenesis. However, only mutations at the Q_P site were examined. Numerous cancer-causing mutations have been reported in other regions of the enzyme (Baysal *et al.*, 2001). Some of these other mutations should also be modeled into the yeast SDH to determine whether a common mechanism is responsible for all SDH-associated tumorigenesis. Although yeast offers numerous biochemical advantages for examining such mutations, it lacks the hypoxia-inducible factor signalling pathway. This limits our ability to follow the effects downstream of oncogenic SDH mutations. Further studies should consider establishing a model system such as *C. elegans*. In *C. elegans*, SDH has a very interesting role in aging and lifespan determination (Ishii *et al.*, 1998; Senoo-Matsuda *et al.*, 2001). The hypoxia-inducible factor signalling pathway is evolutionarily conserved in *C. elegans* and it contains homologs of HIF-1 and prolyl hydroxylase (EGL-9 in *C. elegans*) (Shen *et al.*, 2005). It would be interesting to model oncogenic mutations into the *C. elegans* SDH and characterize their effects on the HIF regulatory pathway.

The involvement of SDH in oncogenesis may be linked with its role in apoptosis because the sensitivity to apoptotic signals and tumorigenesis are often inversely correlated. The SdhB, SdhC and SdhD subunits of the human enzyme have been identified as tumour suppressor proteins and it is postulated that they regulate apoptosis (Albayrak *et al.*, 2003; Gottlieb and Tomlinson, 2005). Transient reductions of SDH activity result in the induction of apoptosis, while a chronic absence of SDH results in tumorigenesis (Albayrak *et al.*, 2003; Ishii *et al.*, 2005). Downregulation of SDH function with small interfering RNAs protects a pheochromocytoma cell line from apoptosis (Lee *et al.*, 2005). In addition, there is evidence to suggest that Tcm62p also regulates apoptosis (Vander Heiden *et al.*, 2002). Yeast is a suitable choice for studies involving the role of SDH in apoptosis because a conserved apoptotic mechanism exists within *S. cerevisiae* and it is thought that apoptosis-mediated aging is similar in yeast and in higher eukaryotes (Madeo *et al.*, 1997). A mutant of the cell division cycle gene *CDC48* as well as cells overexpressing the proapoptotic protein Bax express markers typical of apoptosis (Madeo *et al.*, 1999). The effects of Bax and the *cdc48* mutant expression should be examined in SDH deletion mutants to determine whether the induction of apoptosis is affected. Assays such as membrane staining with annexin V, indicating an exposure of phosphatidylserine in the outer leaflet of the cytoplasmic membrane and terminal transferase dUTP nick end labelling (TUNEL) staining, indicating DNA fragmentation, would be used to examine the progression of apoptosis (Madeo *et al.*, 1997).

ROS are believed to participate in both early and late stages of apoptosis. SDH may function in apoptosis by generating ROS. Yeast cells undergoing apoptosis accumulate ROS and these strains will also be examined for their ability to generate ROS using probes such as dihydroethidium, dichlorofluorescein or lucigenin (Hockenbery *et al.*, 1993; Budd *et al.*, 1997; Li *et al.*, 1999). The SDH inhibitor thenoyltrifluoroacetone (TTFA) induces apoptosis and the production of ROS in cultured cells (Albayrak *et al.*, 2003). TTFA is classical inhibitor of ubiquinone reduction by SDH, as it binds to the ubiquinone binding sites. We will examine whether TTFA and other SDH inhibitors such as carboxin can induce apoptosis in yeast. We will also study the effects of SDH mutations that block inhibitor binding and of tumorigenic SDH mutations on the induction of apoptosis. Such experiments could provide insight into how SDH mutations can result in cancer by inhibiting apoptosis and preventing cell death. This could have significant implications for our understanding and treatment of these disorders.

While many areas of SDH structure and function have been examined in detail, the topic of regulation of enzyme function has been largely overlooked. Emerging evidence indicates that reversible phosphorylation, the most prevalent form of cellular posttranslational modification, is an important means of modulating mitochondrial function (Pagliarini and Dixon, 2006). So far, over 60 mitochondrial phosphoproteins and over 30 kinases and phosphatases have been identified, suggesting that this mechanism of signalling may be involved in regulating many aspects of mitochondrial function. Reversible phosphorylation has been shown to play a role in the regulation of MRC function. Cyclic AMP-dependent phosphorylation of the 18-kDa subunit of complex I stimulates activity of the enzyme and NAD-linked respiration (Papa, 2002), while phosphorylation of subunit II of cytochrome *c* oxidase causes enhanced activity (Miyazaki *et al.*, 2003). SDH is also a potential site of regulation since both the SdhA and SdhD subunits are phosphorylated in bovine heart mitochondria (Schulenberg *et al.*, 2003; Chen *et al.*, 2004). We will examine whether the yeast SDH is phosphorylated and define the stimuli that cause this event. There may be a common stimulus, such as cAMP that regulates multiple components of the MRC. Currently, it is not known whether phosphorylation of SDH is a stimulatory or an inhibitory event. We will examine the consequences of *in vitro* phosphorylation on SDH activity. It will also be important to define the sites of SDH phosphorylation; this will be achieved using methods such as tandem mass spectrometry or Edman sequencing (Chen *et al.*, 2004). Once these residues are identified, they will be mutated and examined for their effects on enzyme function. Reversible phosphorylation of SDH may have several consequences related to its function. For example, it may regulate the ability of SDH to interact with other components of the TCA cycle metabolon (Srere, 1987). Alternatively, this modification may modulate the inclusion of SDH into the MRC supercomplexes that are thought to exist in yeast mitochondria (Boumans *et al.*, 1998). Depending on the their locations, phosphorylation of the subunits may directly modulate catalysis by affecting domain movements that are thought to occur in the catalytic dimer during substrate binding (Ackrell, 2000).

Another mechanism of regulation of SDH may come in the form of differential expression of subunit isoforms that alter the catalytic properties of the enzyme. In *A. suum*, the ability of the enzyme to function as a SDH or as a FRD is dictated by the expression of stage-specific isoforms of Fp and CybS (Sdh1p and Sdh4p in yeast) (Amino *et al.*, 2003; Kita *et al.*, 2003). Two functionally redundant isoforms of Sdh1p exist in yeast (Colby *et al.*, 1998) and in humans (Tomitsuka *et al.*, 2003). The presence of the SdhA isoforms in humans is thought to contribute to the tissue specificity of mitochondrial diseases associated with SDH dysfunction (Tomitsuka *et al.*, 2003; Brière *et al.*, 2005). We have identified homologs for membrane anchor subunits of SDH that are able to functionally replace their wild-type counterparts. However, we have not yet ascertained their roles in SDH regulation and the effects of their expression on enzyme function. Microarray data indicate that the expression of the Sdh3p and Sdh4p homologs is significantly increased when yeast cells undergo sporulation (Chu *et al.*, 1998) or when they are subjected to environmental changes such as temperature shocks, hyper- and hypo-osmotic shock and the progression into stationary phase (Gasch *et al.*, 2000). This suggests that SDH function is altered in response to these conditions. Tcm62p may also be involved in the assembly of these hybrid enzymes since the *Δtcm62* strain exhibits defects in sporulation and cell wall synthesis (Briza *et al.*, 2002). We will examine the effects of growing yeast strains lacking the homologs to a variety of stress conditions. Conversely, we will generate strains that express only hybrid SDH enzymes and test their ability to grow under stress conditions. Results of these experiments will indicate whether membrane anchor subunit homologs confer a growth advantage under these conditions. These strains will also be examined for their sporulation efficiencies since the current evidence suggests that they may play a role in this process. We will also examine the enzymatic properties of the hybrid SDHs by determining turnover numbers for quinone reductase activity and the affinity of these enzymes to quinone binding site inhibitors. The biophysical properties of hybrid enzymes, such as the midpoint potential of the heme will also be examined. This will provide insight into how the hybrid enzyme functions differently compared to the wild-type. Studies involving the Sdh3p and Sdh4p homologs are pertinent to higher eukaryotes since there is some evidence to suggest the existence of isoforms of the SdhD subunit (Hirawake *et al.*, 1999; Tomitsuka *et al.*, 2003).

5.3 Concluding Remarks

Over the past approximately 60 years of research, significant advances have been made in our understanding and knowledge of the structure, function and biogenesis of SDH. The work presented in this thesis adds to this previous knowledge base and provides a platform for future studies. Our characterization of the yeast SDH is pertinent to the understanding of this eukaryotic enzyme and reinforces the notion that *S. cerevisiae* is an excellent model system to use for addressing questions associated with this area of research. More importantly, the studies done in the yeast model system contribute to our understanding of the underlying mechanisms of mitochondrial diseases associated with SDH dysfunction. As the importance of SDH in human health and disease emerges, the need for continuing and evolving studies into the function of this enzyme is highlighted.

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